(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 August 2001 (02.08.2001)

PCT

(10) International Publication Number
WO 01/54486 A1

(51) International Patent Classification[7]: A01H 1/00, C12P 21/00, C07K 1/00, 4/10, 5/00, 7/00, 14/00, A21D 2/00, 6/00, 13/00, G01N 33/10

(21) International Application Number: PCT/US00/02106

(22) International Filing Date: 27 January 2000 (27.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/491,259 26 January 2000 (26.01.2000) US

(71) Applicant *(for all designated States except US)*: KANSAS STATE UNIVERSITY RESEARCH FOUNDATION [US/US]; 1500 Hayes Drive, Manhattan, KS 66502 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: TILLEY, Katherine [US/US]; 2710 Brittany Terrace, #9, Manhattan, KS 66502 (US).

(74) Agent: TRUITT, Tracey, S.; Hovey, Williams, Timmons & Collins, Suite 400, 2405 Grand Boulevard, Kansas City, MO 64107 (US).

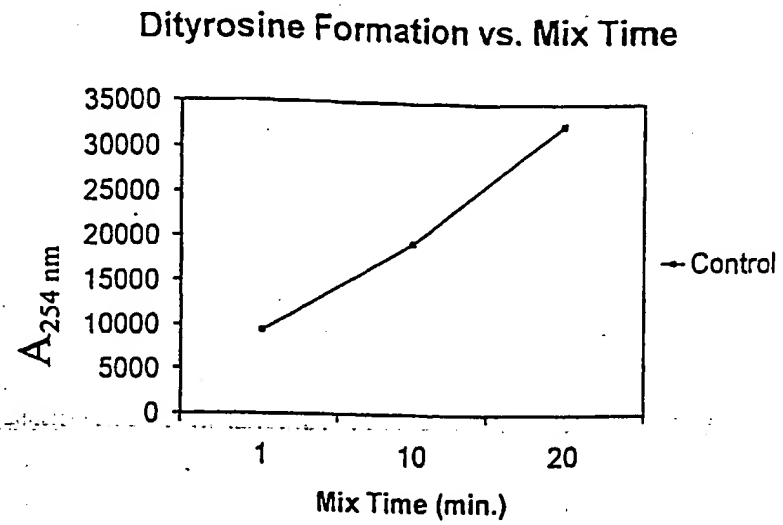
(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PREPARATION AND USE OF POLYMERS CROSSLINKED WITH TYROSINE-CONTAINING PEPTIDES



(57) Abstract: It has been discovered that the formation of tyrosine bonds (and especially dityrosine bonds) has a fundamental and heretofore unrecognized effect upon wheat, wheat flour, wheat doughs and wheat-based end products, i.e., the level of such tyrosine bonds profoundly effects wheat dough properties and product quality. This insight allows effective genetic manipulation of wheat and other grains in order to control tyrosine bond levels in the grain protein, and also leads to methods for monitoring and/or altering tyrosine bond levels during plant growth, flour manufacture and dough formation and processing. In addition, novel polymeric structures made up of polymers (either biopolymers such as proteins or synthetic polymers) can be developed wherein the polymers are crosslinked using tyrosine-containing peptides. The bond-forming peptides typically contain a peptide-bonded tyrosine pair (YY).

WO 01/54486 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 August 2001 (02.08.2001)

PCT

(10) International Publication Number
WO 01/54486 A1

(51) International Patent Classification[7]: A01H 1/00, C12P 21/00, C07K 1/00, 4/10, 5/00, 7/00, 14/00, A21D 2/00, 6/00, 13/00, G01N 33/10

(21) International Application Number: PCT/US00/02106

(22) International Filing Date: 27 January 2000 (27.01.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/491,259 26 January 2000 (26.01.2000) US

(71) Applicant *(for all designated States except US)*: **KANSAS STATE UNIVERSITY RESEARCH FOUNDATION** [US/US]; 1500 Hayes Drive, Manhattan, KS 66502 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: **TILLEY, Katherine** [US/US]; 2710 Brittany Terrace, #9, Manhattan, KS 66502 (US).

(74) Agent: **TRUITT, Tracey, S.**; Hovey, Williams, Timmons & Collins, Suite 400, 2405 Grand Boulevard, Kansas City, MO 64107 (US).

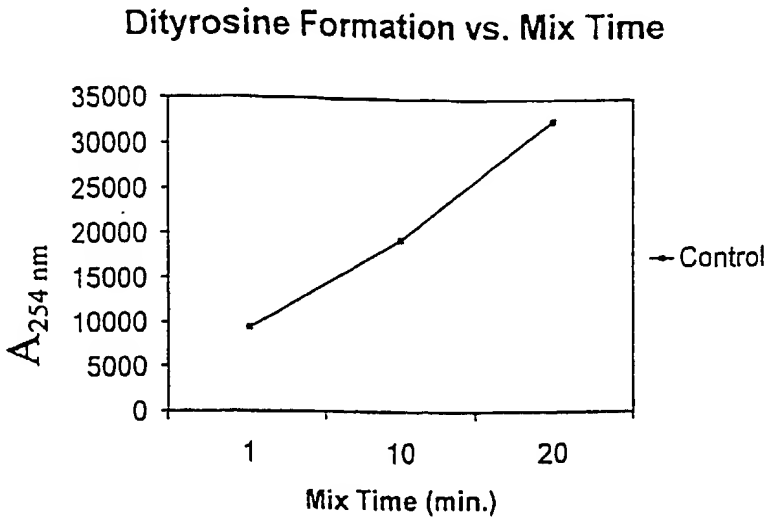
(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PREPARATION AND USE OF POLYMERS CROSSLINKED WITH TYROSINE-CONTAINING PEPTIDES



(57) Abstract: It has been discovered that the formation of tyrosine bonds (and especially dityrosine bonds) has a fundamental and heretofore unrecognized effect upon wheat, wheat flour, wheat doughs and wheat-based end products, i.e., the level of such tyrosine bonds profoundly effects wheat dough properties and product quality. This insight allows effective genetic manipulation of wheat and other grains in order to control tyrosine bond levels in the grain protein, and also leads to methods for monitoring and/or altering tyrosine bond levels during plant growth, flour manufacture and dough formation and processing. In addition, novel polymeric structures made up of polymers (either biopolymers such as proteins or synthetic polymers) can be developed wherein the polymers are crosslinked using tyrosine-containing peptides. The bond-forming peptides typically contain a peptide-bonded tyrosine pair (YY).

WO 01/54486 A1

PREPARATION AND USE OF POLYMERS CROSSLINKED WITH TYROSINE-CONTAINING PEPTIDES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to polymeric structures wherein one or more polymers (either biopolymers such as proteins or synthetic polymers) are crosslinked by tyrosine bonds formed through peptides respectively associated with each polymer or polymer region, as well as isolated peptides useful in deriving such polymeric structures. The invention has particular applicability in the context of grains such as wheat, wherein the crosslinking property of grain protein can be altered by genetically altering a gene which expresses the plant protein in order to cause the altered gene to express a greater or lesser number of tyrosine bond-forming subunits.

The invention also is concerned with monitoring and assessing wheat or flour samples for dough forming potential, monitoring subsequent dough formation and modifying the physical properties of the dough during the course of dough mixing. In practice, the levels of tyrosine, dityrosine, phosphotyrosine, and other tyrosine bonded compounds are measured in growing wheat and in flour to predict dough forming properties, based on the potential level of tyrosine bonds that may be produced during mixing of the flour with water to produce a dough. Furthermore, manipulation of dityrosine or phosphotyrosine content can occur during wheat growth, especially in response to environmental conditions to ensure consistent growth of wheat which provides high quality flour for optimum dough products. The actual levels of tyrosine bonds formed in dough during mixing may also be monitored and manipulated as needed by the addition of oxidizing/reducing agents, free radical scavengers or tyrosine analogs to consistently produce high quality doughs. Additionally, bonds incorporating tyrosine can be analyzed at different stages of end-product formation (e.g. baking).

Description of the Prior Art

In flour dough manufacture, dough is produced by mixing wheat flour and water. Other ingredients (e.g. salt) are added depending on the product being made. Dough made from wheat flour has a viscoelastic property not exhibited by doughs made from other cereals. This viscoelastic property is believed to be derived from gluten protein. The glutenin subunits, one of the two classes of storage proteins which are part of the gluten complex in wheat, are known to directly affect dough formation and bread making quality. Present theories regarding dough formation were developed with the idea that only disulfide crosslinks are involved in the mechanism of gluten structure formation. It was believed that these disulfide bonds were

formed and/or broken and reformed during the mixing process and were ultimately responsible for the characteristics exhibited by a particular sample of dough.

Based on the intended use of the dough, different properties may be desired, i.e., a dough intended to be used for bread may have different desirable properties than a dough made for breakfast cereal processing. Additionally, similar flours used in dough processing may exhibit different characteristics during mixing due to environmental conditions present when the grain used to make the flour was growing or genetic differences. Moreover, some varieties of wheat are less effected by specific environmental conditions than other varieties. Dough manufacture is affected by many different variables and it was heretofore impossible to predict with reasonable accuracy the qualities that any dough will exhibit during mixing based on an *a priori* analysis of the flour or wheat used or knowledge of the conditions under which the wheat was grown.

The addition of oxidizing/reducing agents, free radical scavengers, metal chelating agents, or adjusting dough pH during processing can affect the properties and consistency of the dough as desired. For example, a common modifier and improver of doughs, potassium bromate ($KBrO_3$) is known to have positive effects on dough quality. Due to $KBrO_3$'s dough improving effects, it was a common ingredient in most dough formulas. Unfortunately, $KBrO_3$ has been determined to be potentially carcinogenic at certain levels and its use in bread doughs has been banned in the United Kingdom, Japan and New Zealand. The United States has limited the use of $KBrO_3$ with maximum permitted levels of 50 or 75 ppm. However, following a request from the FDA in 1991, a majority of baking companies have voluntarily stopped using $KBrO_3$.

As a result of processing, dough can become sticky and reduce operating efficiency causing expensive delays and product loss. Alternatively, the dough can be overdeveloped or overworked resulting in low quality products. There is a point in time during mixing of every dough where continued mixing beyond that point results in a dough of inferior quality. Stopping the mixing process prior to that point also results in unacceptable dough quality. What is needed are: methods of monitoring and assessing dough forming potential in developing wheat kernels; modifying dough forming potential in developing wheat kernels; genetically altering wheat cultivars to modify dough forming potential; assessing dough forming potential of a flour prior to processing in order to add proper ingredients/modifiers which will increase quality and contribute to making good end-products and further to precalibrate processing equipment thereby reducing the amount of manipulation required to efficiently produce an optimum dough; methods of monitoring ingredient effects on dough formation; monitoring dough formation during processing so as to assess dough characteristics in a way that consistently results in product optimization; manipulating dough formation during processing to effect optimization of the final dough product; modifying physical characteristics

of polymers including synthetic polymers as well as biopolymers; and genetically altered wheat cultivars which consistently produce high quality doughs despite environmental stress.

Attempts have been made to utilize other grains such as soy in the formation of doughs similar to wheat doughs. These efforts have met very little success, because soy and other grains simply will not form doughs having the characteristic properties of wheat such as stickiness, viscoelasticity and gluten structure. While these results have been well known, the art has never developed a consistent theory as to why these other grains will not form wheat-like doughs. Clearly, any explanation of this conundrum would potentially make it possible to modify such other grains to achieve or at least approximate wheat dough properties.

The art has recognized that wheat glutenins contain tyrosine residues. Many of these proteins have been sequenced and the presence of tyrosine therein is manifest. However, the art has placed no special significance on tyrosine in glutenins or on any effect of tyrosine on the generation of typical wheat and wheat dough properties. For example, PCT Publication of Wesley et al. WO 98/48271 describes a method of monitoring dough properties using infrared spectroscopy to determine glutenin/gliadin ratios. This reference does not, however, teach or suggest determining levels of tyrosine or any tyrosine-related phenomena in doughs. Niemann et al. (Patent No. 4,135,816) teaches a general method for determining total protein content or individual amino acids in flour. Again however, this reference is silent as to the importance or desirability of measuring tyrosine or tyrosine-related phenomena.

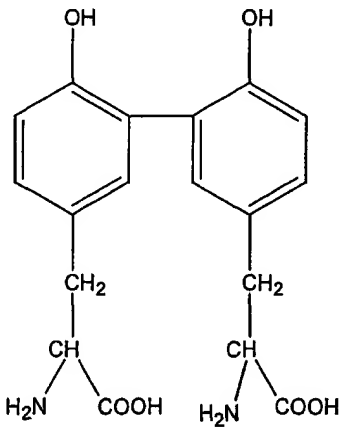
Michon et al., *Biochemistry*, 36:8504-8513 (1997), is concerned only with the oxidation of tyrosine-containing model peptides in ammonium acetate using horseradish peroxidase, followed by analysis of the reaction products using HPLC/mass spectrometry and absorption and fluorescence spectrometry. This reference does not, however, relate the existence of tyrosine bonds to any functional result in plant proteins or other systems.

SUMMARY OF THE INVENTION

I. General Aspects of the Invention

As used herein, the following definitions will apply: "Tyrosine" refers to the tyrosine residues within a peptide or protein chain. "Tyrosine bonds" in the context of plant proteins refers to bonds between a tyrosine residue within a peptide or protein chain and another chemical moiety, free or within a polypeptide, and embraces dityrosine species as well as multiple bonds between respective tyrosine residues and a common bridging moiety. More generally, "tyrosine bonds" refers to bonds other than peptide bonds formed by two peptides, each peptide including therein at least one tyrosine residue and often including a tyrosine pair made up of two peptide-bonded tyrosine residues. These bond forming peptides may be a part of a protein or coupled to a protein, another biopolymer, or a synthetic polymer. "Dityrosine species" embrace dityrosine, isodityrosine, trityrosine, di-isodityrosine, and analogs thereof. "Free tyrosine" refers to the amino acid tyrosine when not within a peptide or protein chain.

"Tyrosine pair" refers to two peptide bonded tyrosine residues (YY), either alone or in a larger amino acid sequence. "Dityrosine" refers to two tyrosine residues linked together by biphenyl or ether linkages. "Dityrosine analytical reference standard" refers to two tyrosine moieties linked through a biphenyl linkage and having the following structure.



"Optimum" with respect to a dough's viscoelastic properties refers to when a dough exhibits desired physical characteristics based on the dough's eventual end-use taking into account the fact that doughs having different eventual end-uses may have different desired viscoelastic characteristics. "Analysis" with respect to tyrosine, dityrosine or phosphotyrosine content refers to any technique for determining tyrosine, dityrosine, phosphotyrosine and/or tyrosine bond content such as amino acid analysis of protein or protein hydrolysates, elucidation and analysis of appropriate nucleic acid sequences, and any other physical analytical methods (e.g. NMR). "Isolated" means altered "by the hand of man" from its natural state., i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a polynucleotide or polypeptide naturally present in a living organism is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein.

The present invention is predicated upon the discovery that a class of tyrosine-containing peptides form tyrosine bonds (as defined above) in the protein fraction of wheat, wheat flour, wheat doughs and final products derived from such doughs, and that such tyrosine bonds have a profound and heretofore unrecognized effect upon final product quality. This discovery makes it possible to test wheat during growth thereof to determine the tyrosine bond level therein and to alter if necessary the growth conditions of the wheat so as to change the tyrosine bond level in the final harvested wheat protein. By the same token, tyrosine bond levels can be measured in flour so as to permit a baker to adjust formulation or baking conditions for optimum results. More fundamentally, wheat may be genetically altered using

known techniques such as site directed mutagenesis in order to increase or decrease the level of tyrosine bond formation.

In addition, the knowledge of the importance of tyrosine bond levels in wheat and the effect thereof on dough and product quality, permits alteration of other grain products (e.g., corn and soy) which do not form wheat-like doughs to incorporate tyrosine bonds into the grain protein thereof. Accordingly, other grains may be genetically altered or otherwise treated to exhibit desired levels of tyrosine bond formation in the protein fraction thereof.

Still further, this knowledge allows the use of tyrosine bond-forming peptides in a wide variety of contexts beyond plant proteins. Thus, isolated peptide crosslinkers can be provided which can crosslink with polymers (either intrapolymer or interpolymer) to yield new non-naturally occuring polymers and composite polymeric structures. Thus, isolated peptides can be used as such crosslinkers wherein the peptides are selected from the group consisting of: (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14. Particularly preferred isolated peptides include YY (i.e., a tyrosine pair), and QQGYYPTS or QPGYYPTS. As used herein, a "reversal" of a peptide refers to a reverse-order amino acid sequence between the amino and carboxyl ends of a given peptide. For example, in the case of QQGYYPTS, its reversal would be STPYYGQQ.

The invention thus includes non-naturally occurring polymers made up of a polymer chain with one or more of the foregoing peptides within or attached to the polymer chain. The class of polymers susceptible to modification is extremely broad, and embraces biopolymers (e.g., proteins, polysaccharides, starches, nucleic acids, lipids) as well as the synthetic polymers described herein. Thus, any naturally occurring wheat or other plant protein made by being modified to include therein the peptide(s), either within the normal protein sequence or as a side chain or end cap to the protein. Similarly, synthetic polymers can be modified with the peptide(s) as internal, side chain, or end cap substituents.

Such non-naturally occurring polymers can be reacted and crosslinked to form composite polymers. The reaction conditions are dependent upon the specific polymers in question, but the level of tyrosine bond formation in the composites can be altered by the presence of a number of reagents. For example, if it is desired to foster an increase in tyrosine bonds in the composite polymers, the crosslinking reaction is aided by the presence of oxidizing agents and/or free radical generators. Typical oxidizing agents are group consisting of $KBrO_3$, ascorbic acid and azo dicarbon amide (ADA), while common free radical generators are selected from the group consisting of the peroxides, peroxidases, and catalases. On the other hand, if a lesser degree of tyrosine bond formation is desired, reducing agents and/or free

radical scavengers may be employed in the crosslinking reaction. The reducing agents include cysteine, glutathione, betamercaptoethanol and DTT, whereas the scavengers are typically selected from the group consisting of BHT, cysteine, glutathione, and t-butyl catechol.

One class of polymeric structures in accordance with the invention includes a pair of discrete naturally occurring biopolymers (e.g., proteins including enzymes, plant proteins, plant storage proteins) coupled together through one or more tyrosine bonds, typically forming dityrosine. The biopolymers in the composite polymeric structure may be the same or different. However, in all cases the tyrosine bond(s) are formed by two peptides respectively associated with each of the discrete biopolymers, and with at least one of the peptides being non-naturally occurring with respect to the corresponding biopolymer. As indicated above, each of the bond-forming peptides individually has from 2 to about 28 amino acid residues therein and including a tyrosine residue; in many cases, the peptides include a tyrosine pair, the latter being made up of two peptide-bonded tyrosine residues. As indicated, the peptides are associated with each biopolymer. In the case of protein biopolymers, the peptide may be within and form a part of the sequence of the protein, or alternately may be attached as a side chain or terminal group on the protein. If necessary or desirable, the bond-forming peptides may be coupled to the corresponding biopolymers through conventional coupling agents.

It is contemplated that the non-naturally occurring biopolymers and crosslinked biopolymer polymeric structures in accordance with the invention will usually be made up of biopolymers selected from the group consisting of plant proteins, such as storage proteins from grains such as amaranth (*Amaranthus hypochondiacus* = *A. leucocarpus, Amaranthus caudatus*, *Amaranthus* cruentus), barley (*Hordeum* vulgare), malting barley, buckwheat (kasha) (*Fagpyrum esculentum*), canary seed (*Phalaris canariensis*), false melic grass (*Schizachne purpurascens*), maize (Zea mays), millet, common millet, (*Panicum miliaceum*), red millet, (*Eleusine coracana*), bulrush millet (*Pennisetum typhoideum*), foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), finger millet (*Eleusine coracana*), pearl millet, bulrush millet, cattail millet (*Pennisetumglaucum*), fonio millet (*Digitaria exilis*), oats (*Avena sativa*), quinoa chenopodium spp quinoa (*Chenopodium quinoa*), rice (*Oryza sativa*), wild rice (*Zizania palustris*), rye (*Secale cereale*), sorghum (Sorghum bicolor), and kamut; wheat and wheat relatives such as *Triticum aestivum, Triticum spp.*, triticale, *Triticum monococcum* (Einkorn), khorasan, *Triticum boeticum, Triticum, monococcum, Triticum dicoccoides* (Emmer), *Aegilops speltoides, Aegilops squarrosa, Triticum durum* (durum), *Triticum turgidum* (rivet), *Triticum turanicum* (Khorasan), *Triticum polonicum* (Polish), *Triticum carthlicum* (Persian), *Triticum aestivum, Triticum aestivum spelta* (Spelt), *Triticum aestivum macha* (Macha), *Triticum aestivum vavilova* (Vavlilovi), *Triticum aestivum tibetanum* (soft winter wheat), *Triticum aestivum vulgare* (common wheat), *Triticum aestivum compactum* (Club), and *Triticum aestivum sphaerococcum* (Shot); oilseeds such as canola (*Brassica napus*), flaxseed (*Linum usitatissimum*), mustard (*Brassica juncea*), safflower (*Carthamus tinctoris*), sunflower

(*Helianthus annuus*), soybean (*Glycine max*), crambe (*Crambe abyssinica*), and meadowfoam (*Linanthes alba*); pulses such as adzuki bean (*Vigna angularis*), anasazi, baby white lima, black beans (turtle), cranberry bean, kidney bean, European soldier, fava, flageollettes, Great Northern, Jacobs cattle, lupini, navy, pinto, prim manteca, rattlesnake, small red, snow cap, white kidney (cannelloni), *phaseolus spp.*, *vigna spp.*, chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), faba bean (*Vicia faba*), grass pea (*Lathyrus sativus*), lentil (*Lens culinaris*), mung bean (*Vigna radiata*), pea (*Pisum sativum*) and guar plant (*Cyampopsis tetragonolobus*); legumes such as alfalfa (*Medicago sativa*), lupin (*Lupinus albus*), cicer milkvetch (*Astragalus cicer*), crownvetch (*Coronilla varia*), and fenugreek (*Trigonella foenum-graecum*); tubers such as potato (*Solanum tuberosum*), cassava, manioc, taro (*Colocasia esculenta*), yam, sweet potato and konjac; as well as the proteins in other plants of uncertain affiliation including psyllium (*Plantago ovata*), carob (*Ceratonia siliqua*), sweet chestnut (*Castanea sativa*), teff, *Eragrostis abyssinica* (*Eragrostis tef*), arrowroot, cowpea, almond, peanut, milkweed, sesame (*Sesamum indicum*), euphorbia (*Euphorbia lagascae*), fennel (*Foeniculum vulgare*), gumweed (*Grindelia camporum*), field peas, horsebean, tapioca, banana, plantain, peas, seaweed, and kelp. Other suitable biopolymers include flax (genus *Linum*), cotton (genus *Gossypium*), industrial hemp (*Cannabis sativa*), wool (sheep fiber), wood (tree fiber), silk (produced by silkworm (moth) larvae *Bombyx mori*), mohair (Merino sheep, angora goats), cashmere (goats), jute (*Corchorus capsularis* or *C. olitorius*), kanaf (*Hibiscus cannabinus*), sugarcane (*Saccharum officinarum*), and sorghum (*Sorghum vulgare*).

Another polymeric structure in accordance with the invention can be made up of only a single naturally occurring biopolymer or synthetic polymer having respective portions thereof coupled together through one or more tyrosine bonds. In this case, the tyrosine bonds are also formed by two peptides respectively associated with the biopolymer or synthetic polymer portions, and with at least one of said peptides being non-naturally occurring with respect to the biopolymer or synthetic polymer. This type of polymeric structure would be possible with relatively large proteins or the like having a conformation permitting portions thereof to come into close adjacency for tyrosine bond formation. Examples of such single biopolymers include enzymes, such as saccharifying enzymes, gluconase, carbohydrase, glucoamylase, protease, pectinase, mannase, urease, cellulase, pentosanase, xylanase, lysozyme, catalase, invertase, isomerase, lipase, hydolase, deaminase, phosphatase, dehydrogenase, oxidase, esterase, lyase, aminoacylase, amyloglucosidase, peroxidase, aspartase, galactosidase, catalase, lactase, debranching enzyme, alcalase, nuclease, polyphenoloxidase, carboxyl peptidase, cellulase, crosslinking enzymes, dehydrogenase, dextranase, diastase, peptidase, peutosanases, metalloprotease, elastase, fatty acid synthetase, hydrolase, demethylase, kinase, β-galactosidase, thermolysin, phosphoglucomutase, synthase, deaminase, pectase, plasmin, renin, polygalachinonase, pullulanase, phosphodiesterase, subtilisin, transferase, and lysozyme.

As indicated above, the invention also includes formation of polymers and composite polymeric structures made up of synthetic polymers having the peptides hereof within the polymeric chain or attached thereto, or in the case of composites plural polymers coupled together through one or more tyrosine bonds. Here again, the tyrosine bonds are formed by two peptides associated with the discrete synthetic polymers, those peptides being the same as the peptide sequences defined previously. Synthetic polymers useful in this aspect of this invention include polymers containing primary and secondary hydroxyl, primary and secondary amino, carboxyl and isocyanate groups; examples of suitable polymers would be the C_2-C_4 polyalkylene glycols (e.g., the polyethylene glycols) and aminated and carboxy-capped derivatives thereof, polysaccharides and their carboxylated and aminated derivatives, the polyacrylates (e.g., polymethylmethacrylate) and derivatives thereof, and the polystyrene, polyethylene and polypropylene copolymers containing hydroxyl, amino or carboxyl functional groups. Also, the peptides may be attached to the individual synthetic polymers in any fashion, such as a side chain or terminal group. These peptide crosslinkers would typically be attached to the synthetic polymers via known coupling agents.

As indicated previously, one important aspect of the invention involves the genetic manipulation of specific plant genes in order to introduce or alter the level of tyrosine bond-forming subunits in proteins expressed by those genes. Of course, wheat is a prime candidate for such genetic manipulation, inasmuch as wheats inherently have such bond-forming subunits in the protein fractions thereof. However, other plants such as those listed above and especially including wheat, soy, corn, rye, oats, triticale, sorghum, rice, and barley can be altered in this manner. Such altered plants can then be used to form wheat-like doughs having the stickiness, viscoelastic and structural properties similar to that of traditional wheat doughs. It will thus be appreciated that, broadly speaking, the invention provides a method of altering a crosslinking property of a protein by genetically altering a gene which expresses the protein in order to cause the altered gene to express a protein having a greater or lesser number of tyrosine bond-forming subunits therein, as compared with the naturally occurring gene.

In the case of a genetic manipulation of a plant protein gene to introduce codons which will express such altered tyrosine bond-forming subunits in the protein, use may be made of conventional gene engineering methods. In the case of wheat, it would normally be preferred to insert the predominantly conserved codon sequence found in wheat glutenin genes and which codes for the subunit QQGYYPTS, namely 5'-caacaaggttactacccaacttct-3', into the selected genes, or to mutate such genes to achieve this sequence. However, owing to the degeneracy of the genetic code, other codons coding for the desired amino acid residues may be employed. Thus, glutamine could be coded for using the codon sequence caa, cag; glycine could be coded for using the codon sequence ggt, ggc, gga, ggg; tyrosine could be coded for using the codon sequence tat or tac; proline could be coded for using the codon sequence cct, ccc, cca, or ccg; threonine could be coded for using the codon sequence act, acc, aca, or acg; and serine could

be coded for using the codon sequence agt, agc, tct, tcc, tca, or tcg. Other specific insertions or mutations would of course be subject to the same analysis.

The invention also comprehends a method of growing a plant having genes in the genome thereof which express proteins including therein (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14. For example, naturally occurring wheat or genetically altered grains described previously are embraced within the applicable plants. The method comprises the steps of periodically analyzing the plant or plant structure during growth thereof to determine the level of tyrosine bonds therein, and in response to such analysis applying a phosphate-containing nutrient to the plant or the soil adjacent the plant. Preferably, a phosphate fertilizer is applied to the soil for this purpose. Such treatment at strategic time(s) during the plant growth has the effect of decreasing the rate of dityrosine and/or tyrosine bond formation in the plant.

II. Wheat Aspects of the Invention

As indicated above, the invention has particular applicability in the context of wheat, wheat flour, wheat doughs and final wheat-based products, and provides techniques for optimizing the foregoing. For example, through use of the methods of the present invention, wheat kernels can be grown which form high quality flours and consistently result in optimum flour dough product preparation despite differences in initial flour quality, environmental stresses which occurred during wheat kernel development, genetic differences or mixing times all of which previously resulted in doughs of dramatically different quality. Overall quality control in wheat development and dough processing can be more tightly controlled through use of the methods of the present invention.

The preferred dough monitoring method includes preparing a dough in the normal fashion and monitoring tyrosine, dityrosine and phosphotyrosine levels as well as tyrosine bond formation. Tyrosine residues can bond and/or form crosslinks between and among other chemical residues or moieties, e.g., tyrosine residues, quinones, hydroquinone, dihydroxyphenylalanine (DOPA), dopaquinone, semiquinones, glutathione (GSH), cysteine, catechols and various carbohydrates. Some of these compounds may also act as a bridge between tyrosine residues in proteins. Structures including tyrosine residues include dityrosine, isodityrosine, trityrosine and other potential structures involving covalent bonds between and among tyrosine residues as well as crosslinks between tyrosine residues and other compounds. Typical tyrosine-bonded chemical moieties found in flours or doughs may include other tyrosine residues, quinones, hydroquinone, dihydroxyphenylalanine (DOPA), dopaquinone, semiquinones, glutathione (GSH), cysteine, catechols and various carbohydrates as well as

other structures which could form tyrosine bonds. However, the most significant tyrosine-bonded moieties consist of tyrosine residues bound to other tyrosine residues through a bonding mechanism other than peptide bonding. NMR analysis has confirmed that the principal tyrosine bonding in wheat, wheat flours and wheat doughs is in the form of dityrosine.

The addition of oxidizing/reducing agents, metal chelating agents, free radical scavengers, free tyrosine or adjusting the dough pH during processing can affect the properties and consistency of the dough as desired. Phosphorylation blocks the formation of tyrosine bonds and therefore interferes with the ability of a flour to form a dough. This phosphorylation can occur during processing by the addition of phosphorous or phosphorous-containing compounds as a dough ingredient or additive, or alternatively, during wheat kernel growth by the application of phosphorous or phosphorous-containing fertilizers to the ground adjacent growing wheat plants. This phosphorous is then taken up by the plant wherein it interferes with the formation of tyrosine bonds. For any given process, predetermined standards for an optimum range of tyrosine bonds will govern the monitoring and any subsequent modification of tyrosine formation in the dough. The monitoring provides continuous feedback indicating the approximate range or levels of tyrosine bonds at individual stages in the process. If there are too many tyrosine bonds, this information is used for example to direct the addition of a specific amount of the amino acid tyrosine, a tyrosine analog, free radical scavengers or metal chelating agents to the dough to prevent over-formation of tyrosine bonds. If this factor is not monitored or tyrosine is not added, continued mixing will cause the dough to become too sticky resulting in reduced processing efficiency. The present invention also allows for mixing to progress past the point in time at which, the dough has an optimum number of tyrosine bonds and the dough exhibits desired viscoelastic properties. If free tyrosine is added to the dough once an optimum range of tyrosine bonds is reached, mixing may continue without a significant subsequent increase in the number of tyrosine bonds and corresponding loss of desired viscoelastic properties. This occurs due to an inhibition of tyrosine residues within the protein or peptide chains binding with other tyrosine residues within the protein or peptide chains brought about by the added free tyrosine occupying the binding sites of the tyrosine residues within the protein or peptide chains. Thus, mixing may continue without a significant corresponding increase in tyrosine bonds and loss of desired viscoelastic properties. Preferably, mixing may continue for up to about 10 minutes after reaching the optimum range of tyrosine bonds while retaining +/- 10% of the desired viscoelastic properties. More preferably, mixing may continue for up to about 20 minutes after reaching the optimum range of tyrosine bonds while retaining +/- 10% of the desired viscoelastic properties. Still more preferably, mixing may continue for up to about 10 minutes after reaching the optimum range of tyrosine bonds while retaining +/- 20% of the desired viscoelastic properties. Even more preferably, mixing may continue for up to about 20 minutes while maintaining +/- 20% of the desired viscoelastic properties. Increasing the pH of the dough will also result in a decrease

in the rate of dough formation and may decrease the rate of tyrosine bond formation. Conversely, if there are not enough tyrosine bonds at a given stage of the mixing process, oxidizing agents may be added which may increase the rate of tyrosine bond formation. This will increase dough quality by causing development of necessary viscoelastic properties. Additionally, decreasing the pH of dough during processing will also affect dough characteristics and may promote tyrosine bond formation.

The preferred method would also include using a computer program configured to achieve a predetermined range of tyrosine bonds in a dough by directing the manipulation and/or addition of additives to the dough during mixing. These steps would be carried out manually or automatically in response to the approximate number of tyrosine bonds found by analysis at any stage in the process. Any suitable analytical procedure could be followed, for example, by fluorescence detection. Following such analyses, the dough could be modified by the addition of the appropriate tyrosine bond formation modifier such as oxidizing agents, metal chelating agents, free radical scavengers, free tyrosine, and pH adjustment. Also, the physical mixing of the dough could be altered as necessary. This procedure could be carried out stepwise until the range of tyrosine bonds is within a predetermined range for a given dough application.

Furthermore, the formation of dityrosine has been found to continue during the actual formation of the end-products. In other words, dityrosine levels continue to increase during baking. Thus, levels of dityrosine can be monitored at different stages of the baking process in order to further optimize knowledge of end-product quality.

As illustrated herein, different cultivars of wheat contain different levels of tyrosine and dityrosine as well as different rates of dityrosine formation, all of which affect later processing steps used to make end-products. Moreover, cultivars generally considered to be of higher quality have or form little or no dityrosine in the developing or mature wheat kernels while cultivars of generally lower quality have elevated levels of dityrosine and greater rates of tyrosine bond formation in developing or mature wheat kernels. However, dityrosine levels can be monitored in wheat kernels as they develop and if dityrosine levels are increasing, phosphorous or fertilizer containing phosphorous can be applied to the ground adjacent the growing plants in order to limit or reduce the rate or limit further dityrosine formation. It has been found that premature dityrosine formation adversely affects dough formation and ultimately end-product formation.

With respect to the wheat storage proteins, it is believed that glutenin has a major role in contributing to the dough forming characteristics of wheat flour. This is because all glutenin proteins have tyrosine (Y) and tyrosine, tyrosine pair (YY) repeats throughout their structure. Usually, these repeats are found in a YYPTS motif or in the generalized peptide sequences, (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; and (3) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$, wherein each X

independently represents any amino acid residue, and the sum of a + b ranges from 0-14. Particularly preferred isolated peptides include YY (i.e., a tyrosine pair), and QQGYYPTS or QPGYYPTS). Different varieties of wheat have different numbers and locations of these repeat sequences in their amino acid profiles. It is these repeat sequences that are believed to be responsible for the majority of dityrosine formation in doughs and dough products. Thus, wheat "quality" in terms of any application for any end product may also be determined by the number and location of these repeat sequences within a particular variety of wheat.

Different cultivars of wheat contain different levels of tyrosine, phosphotyrosine and dityrosine. Moreover, cultivars generally considered to be of higher quality have little or no dityrosine in developing or mature wheat kernels while cultivars of poorer bread-making quality have elevated levels of dityrosine in their developing or mature wheat kernels. However, tyrosine, phosphotyrosine and dityrosine levels can be monitored in wheat kernels as they develop and if dityrosine levels are increasing, fertilizer containing phosphorous can be applied to limit or reduce the rate of further dityrosine formation.

Additionally, environmental conditions such as heat during wheat growth also impact the formation of tyrosine bonds thereby interfering with a flour's ability to form dough. In the case of wheat grown under high-heat conditions, higher levels of dityrosine are formed in the kernels which decreases the quality of doughs made from such wheat. The formation of high levels of dityrosine in the wheat kernels appears to interfere with and/or prevent later formation of dityrosine when such formation is desirable. This premature dityrosine formation may decrease the number of tyrosine residues available for tyrosine bonding or it may just hinder such bonding during dough processing. Different varieties of wheat are effected to different extents by these environmental conditions and thus, certain varieties of wheat are better suited for growth in particular environments. Using methods of the present invention, one can measure tyrosine, phosphotyrosine and dityrosine levels in these flours and therefore be able to account for any differences during subsequent processing. Alternatively, if these levels are being monitored during wheat growth and dityrosine formation is noted, steps may be taken to reduce further dityrosine formation (e.g. by applying phosphorous). Thus, the effects of heat stress can be minimized through monitoring and manipulation of dityrosine formation and wheat producers can consistently grow crops of high quality which produce optimum end-products.

Additionally, the starting flour used to make the dough may be screened to predict the dough forming potential for a particular use prior to initiating any mixing. This screening is done in much the same way as the monitoring of dough during mixing. In this method, the approximate levels of tyrosine, dityrosine, phosphotyrosine and/or tyrosine bonds in a flour sample are measured in order to assess and predict the dough forming potential based on the respective native, naturally occurring amounts of these compounds. Preferably the flour is analyzed to determine the amount of tyrosine therein because the amounts of tyrosine bonds

therein is usually very small. Alternatively, the flour may be analyzed to determine the level of dityrosine. These analyses are normally accomplished by measuring the content of tyrosine and/or dityrosine in the flour. This provides the advantage of a screening technique that is more sensitive than the on-line technique method of analyzing the tyrosine bonds during dough manufacture. Knowledge of the tyrosine, dityrosine, phosphotyrosine and tyrosine bond content of the storage proteins (glutenin and gliadin) in flour to be used in the dough forming process can reduce the amount of on-line manipulation needed to produce an optimum dough for a particular use. Furthermore, this knowledge allows the operators of the machinery used in dough manufacture to precalibrate their mixing apparatus thereby facilitating production of an optimum dough with a minimum of manipulation. As illustrated herein, different cultivars of wheat contain different levels of tyrosine, phosphotyrosine and dityrosine which effects dough characteristics during later processing steps used in forming end-products and ultimately affects the quality of these end-products.

Finally, analysis of the approximate levels of tyrosine, dityrosine, phosphotyrosine and/or tyrosine bonds in developing wheat kernels and/or wheat flour samples contributes to a method of "grading" wheat and/or flour. Flours may be grouped according to such levels found within the storage protein chains. It is believed that the glutenin subunits, and especially the YYPTS or QQGYYPTS motif repeats of the gluten protein chains occupy a more significant role with respect to a dough's viscoelastic properties. However, the gliadin subunits may still be of importance with respect to tyrosine bonds and their effect on a dough's physical characteristics. Flours having tyrosine, phosphotyrosine, dityrosine and/or tyrosine bond levels falling within a certain range would be grouped together and designated as having a certain grade. Alternatively, the numbers and locations of these motifs in the amino acid profiles of the wheat could also be determined and a grading scale developed. The grade would therefore indicate the approximate range of tyrosine and/or tyrosine bonds inherent in the flour or the number and locations of these motif repeats. This would allow users of flour for different applications to choose a flour that has a desired starting amount of tyrosine and/or tyrosine bonds or a particular number or location of these motif repeats which would contribute to the consistent production of high quality end-products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the increase of tyrosine bonds over time during mixing of a dough;

Fig. 2 is a graph illustrating the effect of ascorbic acid on tyrosine bond formation during mixing of a dough;

Fig. 3 is a graph illustrating a control mixograph during dough mixing;

Fig. 4 is a graph illustrating the effect on dough formation by adding free tyrosine during mixing;

Fig. 5 is a graph illustrating the effect of adding phosphotyrosine during mixing of a dough;

Fig. 6 is a graph illustrating dityrosine (designated as DiY) used as a reference standard detected by fluorescence;

Fig. 7 is a graph illustrating the amount of tyrosine bonds present in a flour sample and dough samples mixed for five and ten minutes, respectively, detected by fluorescence;

Fig. 8 is a graph illustrating dityrosine used as a reference standard detected by fluorescence;

Fig. 9 is a graph illustrating the amount of tyrosine bonds detected by fluorescence in a sample of flour;

Fig. 10 is a graph illustrating the amount of tyrosine bonds detected by fluorescence in a second sample of flour;

Fig. 11 is a graph illustrating the derivatized dityrosine used as a reference standard detected by fluorescence;

Fig. 12 is a graph illustrating the amount of tyrosine bonds detected by fluorescence in a sample of derivatized amino acids from a flour sample;

Fig. 13 is a graph illustrating the amount of tyrosine bonds detected by fluorescence in a sample of derivatized amino acids from a second flour sample;

Fig. 14 is a graph illustrating the fluorescent compounds present in a sample of flour;

Fig. 15 is a graph illustrating the fluorescent compounds present in a dough mixed for ten minutes;

Fig. 16 is a graph illustrating the fluorescent compounds present in a flour sample;

Fig. 17 is a graph illustrating the fluorescent compounds including tyrosine present in a dough sample after one minute of mixing in the presence of an additional 1% free tyrosine;

Fig. 18 is a graph illustrating the fluorescent compounds including tyrosine present in a dough sample after five minutes of mixing in the presence of an additional 1% free tyrosine;

Fig. 19 is a graph illustrating the fluorescent compounds including tyrosine present in a dough sample after ten minutes of mixing in the presence of an additional 1% free tyrosine;

Fig. 20 is a graph comparing the peaks from a control dough mixed for ten minutes to a dough mixed for ten minutes with 1% free tyrosine added;

Fig. 21 is a graph comparing the peaks from a sample of flour only and samples from a mixture of flour and water only after five minutes of mixing, after five minutes of mixing and 77 minutes of proofing, and after five minutes of mixing, 90 minutes of proofing and 27 minutes of baking;

Fig. 22 is a graph comparing the peaks from a sample of flour only and samples from a full formula bake after five minutes of mixing, after five minutes of mixing and 27 minutes of proofing, after five minutes of mixing and 90 minutes of proofing, and after five minutes of mixing, 90 minutes of proofing and 27 minutes of baking;

Fig. 23 is a graph comparing the peaks from a sample of flour only and samples from a dough comprising flour, water and 100ppm ascorbic acid taken after five minutes of mixing, after five minutes of mixing and 77 minutes of proofing, and after five minutes of mixing, 90 minutes of proofing and 27 minutes of baking;

Fig. 24 is a graph comparing the peaks from a sample of flour only and samples from a dough comprising flour, water and 45ppm ADA taken after five minutes of mixing, after five minutes of mixing and 77 minutes of proofing, and after five minutes of mixing, 90 minutes of proofing and 27 minutes of baking;

Fig. 25 is a graph comparing the peaks from a sample of flour only and samples from a dough comprising flour, water and 30ppm $KBrO_3$ taken after five minutes of mixing, after five minutes of mixing and 77 minutes of proofing, and after five minutes of mixing, 90 minutes of proofing and 27 minutes of baking;

Fig. 26 is a graph comparing the peaks from three different flour varieties grown under normal environmental conditions;

Fig. 27 is a graph comparing the peaks of KARL 92 wheat grown under different environmental conditions with NWS flour grown under normal environmental conditions;

Fig. 28 is a graph comparing the peaks of TAM 107 wheat grown under different environmental conditions with NWS flour grown under normal environmental conditions;

Fig. 29 is a graph comparing the peaks of flour and an extruded product made with that flour;

Fig 30 is a graph illustrating the detection of dityrosine in maturing wheat kernels;

Fig. 31 is a graph comparing the tyrosine and phosphotyrosine levels from KARL 92 wheat grown under various temperature conditions;

Fig. 32 is a graph comparing the tyrosine and phosphotyrosine levels from TAM 107 wheat grown under various temperature conditions;

Fig. 33 is a graph comparing the dityrosine levels of doughs made with KARL 92 wheat flour which was grown under various temperature conditions;

Fig. 34 is a graph comparing the dityrosine levels of doughs made with TAM 107 wheat flour which was grown under various temperature conditions;

Fig. 35 is a graph comparing dityrosine levels of doughs formed from soft and hard wheats;

Fig. 36 is a graph comparing the levels of dityrosine between Durum flour and doughs made with Durum flour;

Fig. 37 is a graph comparing the levels of dityrosine in doughs having varying levels of cysteine; and

Fig. 38 is a graph comparing the levels of dityrosine doughs having varying levels of glutathione.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The following examples set forth preferred embodiments of the present invention. It is to be understood, however, that these examples are provided by way of illustration and nothing therein should be taken as a limitation upon the overall scope of the invention.

Example 1

In this example, a standard wheat dough was measured during the dough mixing process in order to determine tyrosine bond levels therein.

Materials and Methods

Samples of wheat dough consisting of wheat flour and deionized water were taken during mixograph analysis at one, five, ten, fifteen, and twenty minutes after mixing began. The mixograph method of the American Association of Cereal Chemists (AACC), method 54-40A, was used. Protein was extracted from these samples with 70% ethanol for one hour and then dialyzed. The samples were vacuum dried and submitted for amino acid analysis. The amino acid analysis protocol utilized has been previously described by Malencik et al., 184 *Anal. Biochem.*, 353-359 (1990). The dityrosine used as a reference standard for bonds between tyrosine residues was obtained from the Department of Biochemistry and Biophysics, Oregon State University.

Results

It was determined that the number of tyrosine bonds between tyrosine residues increased steadily during the mixing process. This indicates that bonds between tyrosine residues of gluten proteins are forming during the mixing process. It is believed that the glutenin subunits of the gluten protein chains occupy a more significant role with respect to this increase of tyrosine bonds, however, the gliadin subunits may still be of importance with respect to the increase in tyrosine bonds. As shown in Fig. 1, this steady increase of tyrosine bonds resulted in an increase in the signal detected (absorbance at 254 nm) in the peak representing the tyrosine bond, dityrosine, between one minute of mixing and 20 minutes of mixing. The dough in the experiment began as a regular mixture of wheat flour and deionized water, progressed during mixing to a good quality dough and proceeded to lose its elasticity and become too sticky, eventually proceeding to breakdown as mixing progressed. This data indicates that dough formation in the early stages of mixing and "breakdown" of dough during mixing after peak development are caused by the formation of tyrosine bonds. There does not appear to be any breaking of covalent bonds, but simply the accumulation of tyrosine bonds as mixing continues. This "breakdown" was conventionally thought to be the breaking of covalent bonds (the disulfide bonds) but now appears to be the result of overformation of tyrosine bonds

by the crosslinking of tyrosine residues such that the dough has lost its elastic nature and the mixograph trace appears to be "breaking down".

Example 2

This example measured the effects of a common oxidizing agent, ascorbic acid, on tyrosine bond formation during dough mixing.

Materials and Methods

Samples of wheat dough consisting of wheat flour and deionized water were taken during mixograph analysis at one, five, ten, fifteen, and twenty minutes after mixing began. Prior to mixing, a solution of 5% ascorbic acid was added to the system. Protein was extracted from these samples with 70% ethanol for one hour and then dialyzed. The samples were vacuum dried and submitted for amino acid analysis. The amino acid analysis protocol utilized in Example 1 was utilized in this example. The dityrosine used as a reference standard for tyrosine bonds between tyrosine residues was obtained from the Department of Biochemistry and Biophysics, Oregon State University.

Results

The results of example 2 are illustrated in Fig. 2. Levels of signal detected (absorbance at 254 nm) in the peak representing the tyrosine bond, dityrosine, are much greater than the levels in the control mixograph (Fig. 1) and the formation of tyrosine bonds increases as mixing progresses at a greater rate than in the control mixograph. These results indicate that tyrosine bond formation is enhanced with the addition of agents such as ascorbic acid and these may in turn be used to contribute to modifying the dough forming process in order to provide improved dough based products.

Example 3

This experiment compared tyrosine formation consisting of crosslinks among tyrosine residues between control wheat flour, control wheat flour with 1%(w/v) aqueous free tyrosine added and control wheat flour with 1%(w/v) aqueous free phosphotyrosine added.

Materials and Methods

A dough was prepared by mixing each of the respective flour samples with deionized water. For the samples containing the free tyrosine and free phosphotyrosine, these two latter ingredients were present in the water added to the control flour to produce the dough. Results of this example are given in Figs. 3, 4 and 5.

Results

The mixograph results from the control wheat flour dough are given in Fig. 3. The line marked MP means midline mixing peak and is the point at which the dough is mixed to optimum for breadmaking properties. The lines marked ML and MR indicate points along the midline curve either 2 minutes to the left (ML) or 2 minutes to the right (MR) of the MP. The line marked TP is the envelope mixing peak. "Envelope" refers to the two lines seen, one outlining the top of the curve and the other outlining the bottom of the curve. The peak of the top line is the highest point on it. The lines marked TL and TR are just indicating points on the upper envelope curve either to the left (TL) or right (TR) of the envelope mixing point (TP). The line marked TX is an arbitrary time set for data collection that was set at 8 minutes in this case in order to determine the width of the curve two minutes prior to the end of the analysis. The line marked TT refers to curve tail and refers to the end of the analysis.

Mixograph analyses illustrated in Fig. 4 were performed with free tyrosine incorporated in the deionized water and produced mixograms that did not "break down". In other words, they appeared to have extended tolerance to mixing. These results indicate that the free tyrosine was becoming associated or bonded with the tyrosine within the storage protein structure (mainly in the glutenin subunits) thereby preventing the crosslinking between tyrosine residues within the storage proteins. When compared to the mixograph shown in Fig. 3, it is apparent that the optimum mixing point (TP) is not reached as quickly when free tyrosine is added. ML, TX and TT are also indicated on this mixograph.

In comparison, mixograph analyses performed with phosphotyrosine (shown in Fig. 5) incorporated in the water exhibited properties substantially similar to those obtained with the control flour (shown in Fig. 3) in that they experienced "breakdown". This indicates that the phosphorylated tyrosine does not interact with the tyrosine residues in the storage proteins as free tyrosine does. Bonding of tyrosine through crosslinking between tyrosine residues occurs in the same manner in which it does during the mixing of control flour. As in Fig. 3, lines corresponding to ML, MR, TL, TR, TX and TT are also indicated.

Example 4

This example determined that tyrosine bonds in doughs were measurable by direct analysis with a fluorescence detector.

Materials and Methods

Three 64 μg dough samples consisting of wheat flour and deionized water were taken at zero, five and ten minutes after mixing began. Each sample was analyzed by direct amino acid analysis with fluorescence detection to determine the level of tyrosine bonds consisting of links between tyrosine residues present in each sample. The samples were hydrolyzed and amino acid analysis by HPLC was performed on the underivatized samples. Tyrosine bonds

consisting of crosslinks between tyrosine residues fluoresce when excited at a wavelength of 285 nm and emit light at 405 nm, therefore analyses of the amino acids obtained from dough samples were analyzed under these conditions with a fluorescence detector (a fluorometer). All samples were compared to the dityrosine used as a reference standard which eluted at about 18.9 minutes. Both peaks, the first eluting at about 18.9 minutes and the second eluting at about 22.5 minutes were analyzed by NMR.

Results

Fig. 6 shows the dityrosine used as a reference standard eluting at about 18.9 minutes. Fig. 7 shows the amino acids present in a flour sample compared with dough samples that were mixed for five and ten minutes. Five minutes of mixing was near the optimum mixing point of this flour sample and therefore may represent the "ideal" number of these tyrosine bonds necessary for baking purposes. There is an easily detectable amount of tyrosine bonds formed at this point in mixing as is evidenced by the peaks at about 18.9 minutes and at about 22.5 minutes in the chromatogram.

Fig. 7 also shows the amino acids present in the dough sample that was mixed for ten minutes. Again, the tyrosine bond peaks at about 18.9 and at about 22.5 minutes have increased. This level of tyrosine bond formation is probably indicative of an overformation of tyrosine bonds. The dough has now been mixed beyond its ideal for baking purposes. This sample was taken at a point in mixing that cereal chemists refer to as "breakdown" because the dough does not retain its resistance to extension and is not as elastic as it would be after only five minutes of mixing.

All of these results demonstrate that tyrosine bonds increase steadily during the dough mixing process and are detectable by direct analysis with a fluorescence detector. Additionally, both peaks of interest (the one at about 18.9 minutes and the one at about 22.5 minutes) were determined to be dityrosine of different forms. This is critical because on-line analysis during dough processing is dependent upon direct analysis of the dough while it is in the mixer in order to give timely feedback regarding tyrosine bond formation such that appropriate steps may be taken to manipulate these levels in the dough. This data also demonstrates that tyrosine bond formation occurs continuously during mixing.

Example 5

This example measured the approximate amounts of tyrosine bonds in different wheat flour samples.

Material and Methods

Samples of wheat flour were taken to determine the approximate level of tyrosine bonds therein prior to forming any dough. Knowledge of the amount of tyrosine bonds present

in the flour provides an indication of the crosslink forming potential of a flour sample. It may also indicate the types or extent of modifications that may be necessary in order to produce an optimum dough product. The samples were hydrolyzed and amino acid analysis by HPLC was performed on the underivatized samples. The amino acids were then analyzed with fluorescence at a wavelength of 285 nm and compared to the dityrosine analytical standard which eluted at 19.579 minutes. The amino acid analysis protocol utilized in example 4 was utilized in this example. The dityrosine analytical reference standard was obtained from the Department of Biochemistry and Biophysics, Oregon State University.

Results

The approximate amount of tyrosine bonds in flour samples was determined using fluorescence detection. Fig. 8 illustrates the tyrosine bond standard eluting at 19.579 minutes. Fig. 9 illustrates the approximate amount of tyrosine bonds present in a sample of flour as detected by fluorescence. The peak at 19.432 minutes represents approximately 83 pmol of tyrosine bonds/μg of protein. The tyrosine bond level can then be used to predict the rate at which tyrosine bonds would be formed during the mixing of a dough as well as predicting the amount and type of manipulation that may be needed to consistently produce an optimum dough product. For example, if the flour exhibited a low level of tyrosine bonds (as compared to a control sample standard), the operator of the machinery used to produce a dough would know that a longer mixing time and/or the addition of oxidizing agents would be necessary to produce an optimum dough product. This would also allow any automated machinery to be precalibrated such that the initial tyrosine bond content of the flour would be taken into account when programming mixing times and ingredient additions.

Similarly, Fig. 10 illustrates the approximate amount of tyrosine bonds present in a second flour sample. The peak at 19.412 minutes represents approximately 105 pmol of tyrosine bonds/μg of protein.

Example 6

This example demonstrated a different and more sensitive method of measuring tyrosine bonds in wheat flour.

Material and Methods

Samples of the flour used in Example 5 were hydrolyzed and subsequently derivatized and HPLC analysis was performed on these derivatized amino acids. The derivatiztion procedure of Cohen et al., *Anal. Biochem.*, **211**:279-287 (1993) was used.

Results

Testing on the derivatized amino acids allowed detection of even very low levels of tyrosine bonds. Due to the sensitivity of this testing, it is a preferred method for analyzing flour and predicting tyrosine bond forming potential based on the amount of tyrosine and tyrosine bonds present in the sample. Fig. 11 shows the derivatized dityrosine standard eluting at 59.278 minutes. Fig. 12 illustrates the derivatized tyrosine bonded species from the first sample eluting at 59.261 minutes. The area under the peak represents approximately 83 pmol of tyrosine bonds/μg of protein. Fig. 13 illustrates the derivatized tyrosine bonded species from the second sample eluting at 59.266 minutes. The area under that peak represents approximately 105 pmol of tyrosine bonds/μg of protein. As is apparent from these two samples, flours may inherently have varying amounts of tyrosine bonds. The actual amount of tyrosine and tyrosine bonded species present in a given flour may correlate to the tyrosine bond forming potential of the flour during dough processing. Thus, measuring the initial content of tyrosine and tyrosine bonded species of the starting flour and thereafter measuring and controlling/manipulating the tyrosine and tyrosine bonded species in the dough during processing will result in higher quality end-products with decreased waste.

Example 7

This experiment demonstrated that tyrosine residues link with several components in flour to form fluorescent compounds.

Materials and Methods

First, a sample of flour was analyzed using HPLC followed by fluorescence detection. Results from this sample are given in Fig. 14. Next, doughs made with this analyzed flour were formed by mixing the doughs for 10 minutes and then analyzing these doughs to determine whether other fluorescent compounds were formed. The sample was analyzed using HPLC followed by fluorescence detection. The results from this sample were compared to the flour sample.

Results

Fig. 14 shows the results of the flour sample and Fig. 15 shows the results of the dough sample. In Fig. 15, other fluorescent compounds are evident by the peaks at 12.314 minutes, 17.076 minutes, 22.520 minutes and 36.424 minutes. The tyrosine bond peaks at 18.938 and 22.520 represent dityrosine. This shows that other fluorescent bonds that may be incorporating tyrosine are also being formed during the mixing process which may also affect dough forming characteristics. When combined with the knowledge of the starting tyrosine content, the rate and/or potential for forming bonds incorporating tyrosine could be predicted.

Example 8

This experiment tested the tyrosine bond effects of adding a 1% (w/v) aqueous solution of free tyrosine to flour used to make a dough.

Materials and Methods

Samples of the above-referenced dough were taken at one, five and ten minutes. These samples were hydrolyzed and HPLC performed on the underivatized amino acids. The results were compared to each other and a sample of flour to see which peaks increased as mixing progressed. Results for the flour sample are given in Fig. 16 while the results for the dough samples are given in Figs. 17, 18, and 19 respectively.

Results

As in Example 3, Fig. 4, doughs with 1% (w/v) free tyrosine added have an extended tolerance to mixing. These doughs did not fully form properly mixed doughs during mixograph analysis. The peaks representing tyrosine bonds consisting of bonds between and among tyrosine residues that appear at around 18.8 minutes in these chromatograms and others have almost disappeared. However, the peaks around 22.4 and 24.3 minutes increase dramatically in comparison to the same data from control doughs mixed to the same time but without having the addition of free tyrosine. This is evidence that the compounds of these peaks are incorporating most of the free tyrosine.

The addition of free tyrosine may also prolong mixing times once a predetermined range of tyrosine residues within the gluten storage protein chains have formed bonds. This is due to the free tyrosine bonding with the tyrosine residues within the protein or peptide chains thereby preventing them from forming crosslinks with other tyrosine residues within the protein or peptide chains. To maintain desired viscoelastic properties (consistency, stickiness, elasticity, etc.) beyond the conventional mixing point as determined by conventional mixograph analysis, a quantity of free tyrosine would be added once the range of tyrosine bonds reached a predetermined standard. This free tyrosine would inhibit further binding between tyrosine residues within protein or peptide chains allowing the mixing process to be extended without a change in the viscoelastic properties. Preferably, the mixing time could be extended for about 10 minutes in comparison to the optimum mixing time as determined by conventional mixograph analysis while maintaining +/- 10% of the desired viscoelastic properties of the dough. More preferably, the mixing time could be extended for 20 minutes while maintaining +/- 10% of the desired viscoelastic properties. Still more preferably, the mixing time could be extended for 20 minutes while maintaining +/- 20% of the desired viscoelastic properties of the dough. Similar alterations of the mixing time and maintenance of desired viscoelastic properties of the dough may be accomplished using the other dough modifiers as discussed above.

Example 9

This example compared HPLC peaks in a control dough and a 1% free tyrosine added dough in order to ascertain tyrosine bond levels therein.

Materials and Methods

The above referenced samples were hydrolyzed and HPLC analysis was performed on the underivatized amino acids. Results of this experiment are given in Fig. 20.

Results

Two peaks of interest are represented at 22.447 minutes and 24.557 minutes respectively. As is apparent, the dough with a 1% (w/v) aqueous solution of free tyrosine added has a much higher level of tyrosine bonds in these peaks than in the peaks of the control dough. This indicates that the free tyrosine is being incorporated into the compounds that are forming these peaks and affecting the characteristics of the dough. As in Example 3, Fig. 4 and Example 8, Figs. 17, 18 and 19, the dough supplemented with 1% free tyrosine exhibited an extended tolerance to mixing.

Example 10

This example illustrates one method of determining and setting an optimum range standard for tyrosine bonds in a given wheat flour-containing product. Finding a predetermined optimum standard for such products allows the producer of the product or machinery operator to compare the range or number of bonds present in a production run to that of an ideal product, thus permitting real-time modification of the product.

Materials and Methods

This standard is found by producing the wheat flour-containing product under optimum processing conditions and taking samples at various stages of the production process. These samples are analyzed to determine the approximate range of tyrosine bonds present in the sample at each stage. This could be done with an infinitely large number of samples at an infinitely large number of stages in the process (e.g. every minute, every second, every 1/10th of a second, etc.) in order to provide as narrow of a target or optimum range as possible. The range or number of tyrosine bonds found at each stage of the processing of ideal products is then used as a benchmark to control future processing of that particular product. The number of tyrosine bonds found at each stage of processing is compared to the optimum or ideal number for that stage and any necessary modifications for bringing the number of tyrosine bonds within the optimum range are made, thereby ensuring that an optimum product is made every time.

Preferably, this entire process is done through a computer program configured to direct the processing of any product utilizing tyrosine bonds. For example, the preferred computer program is designed to direct the operator of the equipment to either manually or automatically:

1) Direct random analyses of the material being processed in order to determine the approximate range of tyrosine bonds at that stage of the processing;
2) Compare the analyzed range found to the optimum range for that stage of the process;
3) Direct the modification of the product as necessary with any useful methods to bring the number of tyrosine bonds into the ideal range; and
4) Repeat as necessary or as often as desired for each run.

Results

Comparing the analyzed ranges from each production run with the predetermined ideal standard allows for the production of products that consistently exhibit optimum characteristics. Of course, the ideal range of tyrosine bonds will be more accurate if many samples are taken from many different production runs that result in ideal products. This method is useful for all products utilizing tyrosine bonds in that ideal ranges may be found and used to govern subsequent production of each product. Furthermore, the ideal range may be based on bonds between and among tyrosine residues or between tyrosine residues and other compounds (which may bridge storage protein chains or be storage protein chain substituents) such as quinones, hydroquinone, dihydroxyphenylalanine, dopaquinone, semiquinones, glutathione, cysteine, catechols, various carbohydrates and analogs thereof (tyrosine bonds), all of which may be measured using the methods outlined in the examples above.

Other advantages deriving from the use of a predetermined standard are that modification of the number of tyrosine bonds found at a given stage during any production run based on the ideal range of tyrosine bonds that should be exhibited at that stage results in a subsequent reduction of product that does not meet quality control standards and therefore, a reduction in wasted product. This will also thereby reduce the operating costs associated with wasted product and standardize quality control. Moreover, the parameters controlling the modification of the number of tyrosine bonds can be easily changed should the ideal range ever need to be adjusted.

Example 11

This example demonstrates that tyrosine bonds form during all stages of bread dough manufacture up to and including the baking stage.

Materials and Methods

A dough comprising 99.8 g wheat flour and a 58% level of water absorption was formed. Fifty milligram (50 mg) dough samples were taken at various times during the mixing and immediately placed in 6 N HCl with 1% phenol and evacuated for amino acid hydrolysis. 100 gram pup loaves were prepared using a 90 minute fermentation according to AACC method 10-10B. This method includes five minutes of mixing followed by 25 minutes of proofing (rising or fermenting) called the first punch stage, 52 more minutes of proofing termed the second punch stage, and finally, 13 more minutes of proofing (for a total proofing time of 90 minutes) termed the pan stage. One hundred (100) mg dough or bread samples were taken at the mixing peak (after 5 minutes of mixing), at the second punch stage (after 5 minutes of mixing and 77 minutes of proofing), and after baking for 27 minutes. These samples were immediately flash frozen and lyophilized before being ground with mortar and pestle. Fifty (50) mg of each sample was then hydrolyzed for amino acid analysis by hydrolyzing it under vacuum with 6N HCl with 1% phenol at 115 °C for 24 hours. Hydrolysates were filtered with 0.45 micrometers (μm) glass/nylon syringe filters prior to being lyophilized and resuspended in 500 microliters (μl) deionized water. Twenty (20) μl samples were then injected onto HPLC for analysis and subsequent comparison with a sample consisting only of flour. Results of this example are given in Fig. 21.

Results

The results of Example 11 are given in Fig. 21 which illustrates the HPLC profiles of the amino acid analyses of samples taken from this flour and water only bake. For this example, there were no other ingredients in the dough except for flour and water, thereby allowing dityrosine increases to be attributed to interactions between and among the tyrosine in the flour and water. The line labeled A represents the amino acid content of the flour with no mixing or baking treatment. The line labeled B represents dough that has been mixed for 5 minutes. The line labeled C represents dough that has been proofing (rising or fermenting) for 77 minutes after being mixed for 5 minutes. The line labeled D represents a sample that has completed all stages of bread making and has been baked for 27 minutes. Two peaks of significant interest appear in the samples: the peak at about 18-18.5 minutes and the peak at 21.5-22 minutes which both increase rather dramatically after baking. The peak at 21.5-22 minutes has been positively identified as dityrosine. The peak at 25 minutes also appears to be influenced also but it does not appear to be as significant. This data shows that this dityrosine crosslink is increasing during all stages of bread making and especially during the baking process. Furthermore, this data indicates that dityrosine crosslinking occurs during all stages of dough development (mixing, proofing, etc.) and continues even into the bread making stage (baking). In fact, dityrosine may exert significant effects during the baking process as shown by the significant differences between the stages measured in Fig. 21 at the 21.5-22 minute

mark. The dityrosine level measured at the bake stage (after 27 minutes of baking) is more than double that of the second punch stage which is significantly higher than dityrosine level at the mixing peak and which is also significantly higher than the dityrosine level in flour only.

Example 12

This example is similar to Example 11, and describes an additional test wherein tyrosine bond levels were measured during bread dough manufacture and baking.

Materials and Methods

A full formula dough comprising 99.8 g flour, 67 g water, 2 g compressed yeast (active, dry), 6 g sucrose, 1.5 g salt (NaCl), 3.0 g shortening, and 0.2 g malt flour was formed and subsequently analyzed. As with Example 11, 100 g pup loaves were prepared using a 90 minute fermentation according to AACC method 10-10B. One hundred (100) mg dough samples were taken at the first punch stage pan stage, and after baking for 27 minutes. Dough and bread samples were immediately flash frozen and lyophilized. The freeze dried samples were ground with mortar and pestle and 50 mg of each sample was hydrolyzed for amino acid analysis. The 50 mg samples were hydrolyzed under vacuum with 6N HCl with 1% phenol at 115 °C for 24 hours. Hydrolysates were filtered with 0.45 µm glass/nylon syringe filters before being lyophilized and resuspended in 500 µl deionized water. Twenty (20) microliter samples were injected onto HPLC for analysis and subsequent comparison with a sample consisting only of flour. Results of this experiment are illustrated in Fig. 22.

Results

As in Example 11 (Fig. 21), dityrosine crosslinks were shown to be increasing during all stages of bread making and especially during the baking process. This is evident by the increases in the peaks at approximately 19 minutes and approximately 21-22 minutes which were found during each successive stage of the bread making process. This example differed from Example 11 in that it represents a full formula bake, meaning that it includes all of the necessary ingredients for bread making and a good quality loaf of bread resulted (in contrast to the loaf produced by the flour and water bake of Example 11, which was of low quality). The increase of dityrosine is apparent as the dough goes through the various mixing and baking stages and these increases appear to be greater than the increases seen in the flour and water only bake. This indicates that use of a full formulation dough enhances dityrosine crosslinking and contributes to end-product quality.

Example 13

This example illustrates the effect of ascorbic acid on dityrosine bond formation during bread mixing and baking processes.

Materials and Methods

The same dough formula described in Example 11 (comprising flour and water only) was used for this example. However, 100 parts per million (ppm) of ascorbic acid was added to the water prior to mixing the water with the dough. Following formulation of the dough, the same analysis procedure used in Example 11 was followed. Again the samples were also compared to HPLC analysis of flour only.

Results

Results of this example are given in Fig. 23 which illustrates the effect that ascorbic acid has on dityrosine during mixing and baking. As is apparent, ascorbic acid exerts its major influence on dityrosine during the baking process. The peak most influenced is the one at 21-22 minutes while the other peaks are not significantly effected. When compared with the results from Example 11, the addition of ascorbic acid appears to have had little or no effect on the peak at approximately 19 minutes while greatly increasing (more than quadrupling) the dityrosine peak for all samples at approximately 21-22 minutes. Interestingly, the samples taken after 5 minutes of mixing and at the second punch stages do not show appreciable differences at any peak including the peak at 21-22 minutes. However, a significant difference is shown after baking the dough. Thus, ascorbic acid appears to increase the number of dityrosine bonds significantly, especially during the baking process.

Example 14

This example illustrates the effects of ADA when added to the dough during mixing.

Materials and Methods

A dough was formed with the same levels of flour and water as in Example 11. Additionally, 45 ppm ADA was added to the water before the water was added to the flour to make the dough. The analysis procedure of Examples 11 and 12 was then followed to produce the results given in Fig. 24.

Results

Fig. 24 illustrates that ADA exerts an effect on dityrosine content when added to the mixing process of a dough. Significant effects are shown at the peak eluting at approximately 21-22 minutes while the other peaks are not significantly effected. Comparing Example 14 (Fig. 24) with Example 11 (Fig. 21), it is apparent that ADA greatly increases the dityrosine content of the dough during mixing and at the second punch stages of dough development. When compared with the results from Example 11, the addition of ascorbic acid appears to have had little or no effect on the peak at approximately 19 minutes while greatly increasing

the dityrosine peak for all samples at approximately 21-22 minutes. Interestingly, the samples taken after 5 minutes of mixing and at the second punch stages do not show appreciable differences at any peak including the peak at 21-22 minutes. However, a significant difference is shown after baking the dough. Thus, ascorbic acid appears to increase the number of dityrosine bonds significantly, especially during the baking process.

Example 15

This example shows the influence $KBrO_3$ exerts when added to a flour and water only mixture.

Materials and Methods

This example was carried out using the same formula and procedures used in Example 14 with the exception that 30 ppm of $KBrO_3$ was added to the water before mixing it with the flour instead of the 45 ppm of ADA. Again, samples of the dough were taken after 5 minutes of mixing, at the second punch stage after 5 minutes of mixing and 77 minutes of rising and fermentation and at 27 minutes into the baking stage. These samples were analyzed as described in Example 11. Results of this example are given in Fig. 25.

Results

As shown by Fig. 25, the incorporation of $KBrO_3$ greatly effected the dityrosine peak at 21-22 minutes for all samples. The dityrosine peak at 21-22 minutes from the baking sample is 2.5-3 times greater than any of the other examples containing oxidizing agents. In contrast to Figs. 11-14, the other peaks are not as significantly effected. This data indicates that the action of $KBrO_3$ as an improving agent lies in its ability to increase the amount of dityrosine crosslinks formed during baking.

Example 16

This example compared three different flours, NWS control flour; KARL 92 control flour; and TAM 107 control flour. NWS flour and KARL 92 flour are generally regarded as flours which produce good quality bread while TAM 107 is generally regarded as being a flour which produces medium to poor quality bread.

Materials and Methods

Each of the above referenced flours were tested for their amino acid profile in order to determine differences in dityrosine content and to correlate this measured dityrosine content with known flour properties. Wheat of each of these varieties was grown in a field under normal conditions, harvested, and tested for their amino acid profiles. The results of this example are given in Fig. 26.

Results

As shown in Fig. 26, TAM 107 has an inherently higher level of dityrosine as shown by the peak at 21-22 minutes than either NWS flour or KARL 92 flour. Because TAM 107 is regarded as being of lower quality than KARL 92 or NWS flour with respect to bread making quality, it is interesting to note that TAM 107 has a higher dityrosine level than either of the other two flours. Therefore, these results indicate that TAM 107 may form too many dityrosine bonds during kernel development. Consequently, these previously formed dityrosine bonds either prevent or do not allow the proper formation of a good quality dough. Ideally, most dityrosine bonds should form during the mixing and baking processes as the flour proteins interact with each other during mechanical processing (mixing) and as a result of ingredient interaction and not in the kernels during seed development. Thus, when selecting flours for particular dough applications, one should be conscious of native dityrosine levels in certain flours. These levels can then either be accounted and compensated for during processing or wheat with proper levels of dityrosine for certain applications can be selected. Alternatively, genetic differences contributing to dityrosine formation in kernels during seed development could be determined and manipulated (e.g. through phosphorylation) so as to produce flours which form certain levels of dityrosine at certain times during kernel development, dough mixing, dough development or baking. Furthermore, timing differences in dityrosine formation may be more or less desirable for different dough applications and end uses thereby permitting better flour selection and processing procedures based on these differences which will result in the consistent formation of more high quality end-products.

Example 17

This example compared dityrosine levels from a good bread-making quality flour, KARL 92, when grown at various temperatures.

Materials and Methods

KARL 92 flour and NWS flour were grown under normal field conditions. Additionally, one sample of KARL 92 flour was grown in a greenhouse at 20°C at 10 days post anthesis while another sample was grown in a greenhouse at 40°C at 10 days post anthesis. Results of this example are given in Fig. 27.

Results

Fig. 27 verifies that temperature conditions present during kernel development have an effect on the ultimate bread-making quality of flours. As shown by the peak at approximately 21-22 minutes, the KARL 92 sample grown at 40°C had increased levels of dityrosine bonds formed during kernel development. As explained in Example 16, this increased level of dityrosine bonds formed during kernel development prevents or does not

allow the proper formation of dityrosine bonds during the mixing and baking processes. The sample grown at 20°C did not have significant differences when compared to NWS and KARL 92 control flours grown under normal environmental conditions. With respect to the sample grown at 40°C, the increase shown at the peak at approximately 21-22 minutes is not as significant as some of the other peak differences shown in the previous examples. Moreover, the resultant dityrosine level is only slightly higher than the level exhibited by TAM 107 grown under normal environmental conditions (see Fig. 26). This indicates that KARL 92 is somewhat "heat-resistant" and can therefore be grown in a wider variety of environments and under a wider variety of environmental conditions without significantly compromising final wheat flour quality. Thus, with knowledge of the environmental conditions under which a wheat sample is grown, one can predict dityrosine levels formed during kernel development and hence determine the dough forming and bread making qualities which will be possessed by products incorporating that particular variety of wheat. Moreover, wheat known to be "heat-resistant" can be selected for and perhaps genetically combined with less heat-resistant varieties in order to increase their heat-resistance. Additionally, wheat varieties known to be heat resistant can be used in hotter climates.

Example 18

This example compared dityrosine content of TAM 107 flour grown at various temperatures.

Materials and Methods

Samples of NWS control flour grown under normal environmental conditions, TAM 107 control flour grown under normal environmental conditions, TAM 107 flour grown at 20°C at 10 days post anthesis and TAM 107 flour grown at 40°C at 10 days post anthesis were analyzed for dityrosine content. Results of this experiment are given in Fig. 28.

Results

This example can be compared to Example 17 wherein KARL 92 wheat was grown at various temperatures and analyzed for dityrosine content. Fig. 28 illustrates that TAM 107 is less heat-resistant to tyrosine binding during kernel development than the KARL 92 flour shown in Fig. 27. The peak at 21-22 minutes demonstrates that the TAM 107 sample grown at 40°C at 10 days post anthesis was greatly effected by the heat as evidenced by the large increase in the amount of dityrosine bonds illustrated by that peak. Additionally, the sample grown at 20°C at 10 days post anthesis had a slight increase in its dityrosine peaks when compared with the two control samples. The peak at 25 minutes does not appear to change significantly with increased temperature. This data indicates that TAM 107 does not resist the influences of increased environmental temperatures as well as KARL 92. The TAM 107

samples decrease in bread-making quality due to the premature formation of dityrosine which occurs at a much greater rate than the KARL 92 samples grown under these various environments. This premature formation may inhibit or prevent the formation of dityrosine during dough processing and baking when it is needed to produce good quality products. Alternatively, the formation of dityrosine at this point may not leave enough available tyrosine residues for later binding during the dough processing and mixing stages. Thus, it appears that temperature-related stress and a subsequent decrease in bread making quality can be correlated with the wheat variety's ability to resist over-formation of dityrosine bonds in the wheat kernels during development.

Example 19

This example determines the effects of extrusion processing on dityrosine formation.

Materials and Methods

Extrusion was performed with a TX-52 twin screw extruder (Wenger Manufacturing, Sabetha, Kansas). Flour was fed into the extruder at a rate of 100 kilograms/hour. The preconditioning mixing cylinder speed was 316 rpm and the extrusion screw speed was 445 rpm. No steam was used and the water flow in the extruder was 21.2 kilograms/hour. Barrel jacket conditions were the following: second head: 27°C, third head: 27°C, fourth head: 130°C, fifth head: 130°C, sixth head: 125°C, seventh head: 125°C. One 5.5 millimeter dye and four knife blades were used. Extrudate was collected and lyophilized. The extruded product was very similar in structure and textural properties to a puffed "cheese curl" snack or to a piece of starch based packing material. Fifty (50) mg of flour or 50 mg of the extrudate that had been lyophilized and crushed in a mortar and pestle were hydrolyzed for amino acid analysis using the same procedure previously employed in this work. The HPLC method of amino acid analysis with fluorescence detection was also the same procedure used previously in this work. Fig. 29 illustrates the HPLC chromatograms of this flour and the extruded product made from that flour.

Results

The flour sample exhibits a very small peak at 22.5 minutes, indicating very little dityrosine present in this control sample. Extruded samples processed with this flour contain an increased level of dityrosine. Thus, dityrosine must be formed during the extrusion process. Moreover, the same conclusions that were drawn for the dough being mixed can be drawn from the flour being mixed and extruded using extrusion processes. It appears that dityrosine is being formed during this process at a rate higher than that detected in a standard dough that was mixed for ten minutes, however, the level of dityrosine in the finished product (extrudate) is only about one-third that of the dityrosine found in a full formula fully baked bread sample.

Thus, dityrosine continues to be formed at all stages of the production process, regardless of whether this process is through traditional mixing and baking or through other methods such as extrusion.

Example 20

This sample illustrates the detection of dityrosine in maturing wheat kernels. Kernels of wheat were harvested from wheat plants of the KSU experimental line KS97HW131, which is a quality noodle making variety of wheat but is not a good bread making variety of wheat. It is a cross between the cultivars Ike and KS91HW19.

Materials and Methods

The above referenced wheat plants were grown in the green house and developing kernels extracted from the head of the plants at 5, 10, 15, and 25 days post anthesis (fertilization/flowering of the wheat plant) as well as after complete maturation of the grain. The kernels were kept on dry ice and then the bran layer and the germ were excised with a razor blade. The remaining endosperm chunks (where flour comes from) were hydrolyzed for dityrosine analysis as described previously. All dityrosine analyses were then performed as previously described. Results of this example are given in Fig. 30.

Results

Fig. 30 demonstrates that there was very little dityrosine present at 5 days post anthesis. At 10 and 15 days post anthesis, an increased amount of dityrosine was present and the levels for 10 and 15 days were very similar. At 25 days post anthesis, a significant increase in dityrosine was detected. This is the point in the development of the kernel where the glutenin polymers have been shown to form. An increased level of dityrosine, which is responsible for crosslinking in these glutenin polymers is detected. At full maturity, the kernels have an even greater amount of dityrosine present. As noted above, this cultivar of wheat is not good for bread making. Accordingly, it has an increased level of dityrosine present in the kernel (as shown before with the KARL 92 and the TAM 107 grown under increased temperatures). As previously demonstrated, the more dityrosine present initially in the flour, the lower the mixing/bread making quality. Thus, dityrosine bond formation can be measured during wheat kernel development. This would allow a producer to manipulate the levels of dityrosine by any means and monitor the dityrosine formation and effects of manipulation in the wheat kernels as they mature. Analyses of these types will allow a grower of wheat to make the correct decisions about when to fertilize and how much to fertilize. Additionally, these results, along with the results from Examples 16, 17 and 18 demonstrate that better bread making wheat varieties have less dityrosine in the developing kernels. However, this example also

demonstrates that wheat varieties which are not good for bread making may be good varieties for other applications (i.e. noodle making).

Example 21

This example compared tyrosine and phosphotyrosine levels from two different wheat varieties grown under various temperature conditions. The wheat varieties tested were KARL 92 and TAM 107.

Materials and Methods

A sample of each of the above referenced varieties was grown under natural temperature conditions for all stages of growth (grown under natural conditions in a field) and used as a control, another sample of each variety was grown under green house conditions of 25°C during the day and 20°C during the night until 10 days post anthesis (dpa). At this point, the greenhouse samples were divided into two groups per variety. One group of each variety was then grown at 20°C and the other was grown at 40°C until harvest.

After harvest, samples from each variety and from each condition were analyzed for tyrosine and phosphotyrosine content using Approved Methods 08-01 (AACC 1995) using an FP-2000 Nitrogen/Protein Analyzer (Leco Corp., St. Joseph MI). Nitrogen values were converted to protein by multiplying by a conversion factor of 5.7 (a standard nitrogen conversion for wheat). Flour weights were calculated such that each flour sample used contained 1 mg of total protein. The flour was placed in separate hydrolysis tubes with 1 ml of methane sulfonic acid (4N) (Pierce, Rockford, IL) containing 0.2% 3-(2-Aminoethyl)-indole-HCl. The hydrolysis tubes were evacuated and placed in a dry heating block for 3 hours. Hydrolysis longer than 3 hours resulted in a complete loss of phosphotyrosine. This loss was likely due to the heat labile phosphoester bond contained in phosphotyrosine. After hydrolysis, the hydrolysate was chilled at 4°C. 100 µl aliquots were taken from each hydrolysis tube and neutralized with an equal volume of 8N sodium hydroxide (NaOH). Each sample was then thoroughly mixed. After mixing, a 20 µl aliquot of the neutralized hydrolysate was added to 80 µl of borate buffer (Waters AccQ-Flour™ Reagent Kit). This mixture was completely mixed using the vortex genie. After mixing, the solution was centrifuged at 10 X 1,000 g for 10 minutes. The supernatant (80 µL) was placed in a mini-HPLC vial and derivatized with 20 µl of 6-aminoquinolyl-N-hydrozysuccinimidyl carbamate (prepared according to directions provided in Waters AccQ-Flour™ Reagent Kit) and capped. The derivatized sample was allowed to stand for two minutes at room temperature. After the two minutes had passed, the samples were placed in a dry heating block (55°C) for 10 minutes to degrade byproducts of the derivatization reaction. Finally, the derivatized samples were ready to be analyzed by HPLC.

The Hewlett-Packard model 1100 HPLC was used to perform all chromatographic analysis. The 1100 model contains a vacuum degasser, quaternary pump, auto-injector,

thermostatted column, diode array detector, and a fluorescence detector. A linear gradient along with a Luna 5μ C(18)2 250 x 460 mm reversed phase column (Phenomenex, Torrance, CA,) was used to separate the sample constituents. Injection volume was 20 μl. The solvents used in the linear gradient are denoted as A and B. Solvent A was a phosphate buffer prepared by dissolving 6 ml of 85% phosphoric acid in 1 liter of deionized water and titrated to a pH of 2.85 with 2N NaOH. Solvent B was a mixture of acetonitrile and deionized water (60%/40% respectively). The linear gradient begins with solvent B at 2% and increases from time zero to 100% over 45 minutes. The column temperature was set at 38°C. Fluorescence detection parameters included an excitation wavelength 250nm and an emission wavelength of 395 nm.

Phosphotyrosine and tyrosine standards were used to determine elution times for each amino acid. Phosphotyrosine had an elution time of approximately 13.2 minutes, and tyrosine had an elution time of approximately 17.5 minutes. Results of this Example are given in Figs. 31 and 32.

Results

The levels of phosphotyrosine (PY) increase when the wheat plants are exposed to 40°C temperatures at 10 dpa in both the KARL 92 and the TAM 107 samples. Fig. 31 shows the phosphotyrosine (13.2 min peak) and tyrosine (17.5 min peak) levels for the KARL 92 samples and Fig. 32 shows the phosphotyrosine and tyrosine levels for the TAM 107 samples. In Fig. 32, the first chromatogram is shifted slightly to the right. The PY and tyrosine (Y) peaks are marked on that chromatogram. This shift can occur during HPLC analyses between runs especially if it is the first sample analyzed in an experiment as this was. As long as the pattern is the same and all the appropriate peaks are present, as in this case, it is legitimate to compare the chromatograms.

The level of PY in the TAM 107 control sample (field grown) is much greater than the level of PY in the KARL 92 control sample (field grown), while the levels of Y in both field grown samples are similar. The levels of PY and Y in the KARL 92 sample appeared to decrease when the sample was exposed to 20°C at 10 dpa. The level of PY and Y present in the flour of KARL 92 increased significantly when the plants were exposed to 40°C 10 dpa.

The level of PY in the TAM 107 samples increased when the plants were exposed to 20°C and 40°C at 10 dpa, and the PY levels appeared to be greater in each of the TAM 107 samples than the KARL 92 samples from the same temperature conditions. The tyrosine levels in the TAM 107 sample did not appear to increase significantly, whereas there was an increase in the tyrosine levels in the KARL 92 samples exposed to 20°C and 40°C conditions.

These analyses show that Y and PY can be detected in flour samples and that levels of each vary depending upon the environmental conditions to which they are exposed. Of primary interest is the fact that both cultivars of wheat show increased levels of PY when heat stress is high (40°C). It appears that the stress response in wheat is to phosphorylate tyrosine residues

in the endosperm proteins of wheat. Tyrosine levels also increased in the KARL 92 sample, but did not appear to be affected as greatly in the TAM 107 sample.

Example 22

This example compares mixograph analyses taken from dough after 10 minutes of mixing and dough made with flour from each sample from Example 21. These analyses determined the mixing quality (and potential breadmaking quality) of each sample.

Materials and Methods

Mixograph analyses were performed on the above referenced dough and flour samples as previously described in Example 3.

Results

The mixographs demonstrate that the KARL 92 (field harvest) has the best quality in terms of dough mixing parameters. There was a gradual increase in the curve until a high peak was reached and then there was a gradual decrease in the curve. The graph of the curve also stayed fairly wide as it decreased. This is a measurement of "tolerance" of a dough to overmixing. The KARL 92 (20°C) sample showed a decrease in the peak height, making it a slightly lower quality mixograph. The mixing peak of the graph was not as high a the field harvest sample. The KARL 92 (40°C) had a fairly good front half of the curve and a nice peak, but the back half dropped off very quickly into a much thinner curve. This result means that the dough has less tolerance and is much poorer than the field grown sample or the 20°C sample.

The TAM 107 sample (field grown) started off with a poor mixograph. There was very little increase to the peak and the peak was low. Then it tapered off with a fairly thin tail. The TAM 107 (20°C) sample hardly had a peak at all and was almost a flat graph. Because there is no distinct peak, these results showed very little elastic dough structure being built up and no strength. The TAM 107 (40°C) sample was extremely poor. There was a very quick little peak that occurred, but it was low and it tapered off quickly to a very thin graph, exhibiting no tolerance.

Thus, mixograph analyses confirm that mixing quality determined by these methods mirrors that determined by tyrosine and phosphotyrosine analyses. Additionally, flours known to have higher levels of phosphotyrosine or dityrosine in the flour are of lower breadmaking quality as are flours with lower levels of tyrosine in the flour.

Example 23

This example compared dityrosine levels from doughs containing two different types of flour after the doughs were mixed for 10 minutes. The flour used to make the doughs came from the samples harvested in Example 21.

Materials and Methods

Samples of TAM 107 and KARL 92 were grown under normal field conditions or in a greenhouse at 25 °C during the day and 20 °C during the night until 10 dpa and thereafter each sample was grown at either 20 °C or 40 °C until harvest.

Mixograph analyses (10 minutes of mixing) were performed for doughs made from each flour sample. The doughs were then placed in liquid nitrogen and lyophilized immediately after mixing. Dityrosine analyses were performed as described previously.

Results

The results of this example are given in Figs. 33 and 34. As shown by Fig. 26, virtually no dityrosine is detected in KARL 92 samples grown under normal field conditions. However, as shown by Fig. 26, dityrosine is detected in the corresponding TAM 107 sample. All samples of flour grown under heat-stressed conditions exhibit some dityrosine (see Fig. 27 and 28). Similarly, dough made from such flour also exhibits increased dityrosine when compared to flours derived from wheat grown under normal environmental conditions. As shown by Figs. 33 and 34, doughs derived from TAM 107 grown under heat-stress contain much larger amounts of dityrosine as evidenced by the significantly larger peaks.

As previously shown, flour derived from TAM 107 wheat samples contains more dityrosine than flour derived from higher quality wheat samples such as KARL 92. Additionally, these two wheat varieties react differently to heat stress in that TAM 107 samples exhibit increasing levels of dityrosine as temperature conditions present during wheat growth increase. Thus, these results verify that doughs made from these flours also differ in their dityrosine levels. Very little dityrosine forms during the mixing of the higher quality wheat variety (KARL 92) even when the plants from which the flour was obtained were exposed to increased temperatures during kernel development. However, increasing amounts of dityrosine form during the mixing of flour derived from TAM 107 wheat which is generally considered to be of lower bread-making quality. The more dityrosine formed during mixing, the poorer the bread-making quality. The KARL 92 flour samples grown at 20 °C and 40 °C had approximately the same amount of dityrosine after ten minutes of mixing as the standard flour analyses discussed in Examples 16 or 17. Dough made from the KARL 92 sample grown at 20 °C also had approximately the same level of dityrosine in the second peak (the peak which occurs at approximately 25 minutes) as the standard flour analyses discussed in Examples 16 or 17.

Moreover, these results indicate that flour used to make good quality mixed doughs (mixed by standard Mixograph analyses) have a range of dityrosine, detected by the methods explained in the preceding examples, which lies between 0-0.25 LU. More preferably, the dityrosine ranges between 0-0.15 LU and still more preferably between 0-0.05 LU. Thus, poorer quality doughs are those which have dityrosine levels greater than 0.25 LU. Additionally, good quality flours should have little or no detectable dityrosine prior to mixing. Once again, there should not be too many dityrosine bonds already present in a good flour because a certain number of these bonds need to form during mixing and not before, to provide the right dough structure. Also, too many dityrosine bonds should not form during the initial mixing stage for similar reasons pertaining to dough structure. This is because even more dityrosine bonds form during the rest of the bread-making process (i.e. kneading, fermentation or proofing, and baking). If too many dityrosine bonds are formed in the mixing process then fewer tyrosine residues are available for dityrosine bonding necessary in the other bread-making steps and a good-quality dough structure cannot be formed. Thus, all of the steps of bread-making are critical in forming the right texture and internal structure of the bread and for providing the opportunity for the dough to expand during the proofing process and in the oven during baking. It is becoming apparent that the right number of dityrosine bonds must form during each of these stages to get a good-quality loaf of bread. Clearly, too many dityrosine bonds at any stage of the process is detrimental and leads to lower quality loaves of bread.

Example 24

This example manipulated the phosphorous levels during plant development under normal conditions in order to effect dityrosine levels in glutenin proteins.

Materials and Methods

Wheat plants from the cultivar (Chinese Spring) were tested in this experiment. The Chinese Spring variety was germinated in vermiculite for 5-7 days until sprouting but was then immediately planted. All subsequent growth for this variety took place under greenhouse conditions set at 25 °C during the day and 20 °C during the night. At anthesis, the heads of the growing plants were tagged to designate the date of anthesis and the plants were fertilized at 2, 4, and 6 weeks after planting as described in *Quality of Hard Red Winter Wheat Grown Under High Temperature Conditions During Maturation and Ripening*, Gibson et al., 75*Cereal Chem.*, 421-427 (1998). All fertilizers were of the N-P-K type in the following concentrations: 10-60-10 and 10-15-10. A control set of plants which received no fertilizer were also tested for each variety. Wheat kernels from the group receiving no fertilizer and from the experimental groups were removed at 20 dpa. All wheat kernels were then processed for dityrosine analysis as previously described. Thus, this experiment determined the effects of phosphorous on dityrosine formation.

Results

Flours made with wheat grown in the presence of increased phosphorous levels (from the N-P-K fertilizers) have decreased levels of dityrosine in the kernel. Flours made with wheat grown without increased phosphorous levels have increased levels of dityrosine. Dityrosine formation for the groups receiving the highest phosphorous levels was substantially eliminated. Results showing the area under the dityrosine peak are given below in Table 1. These results show that the topical addition of fertilizer containing phosphorous at increasing levels to the ground adjacent growing plants decreases the levels of dityrosine in the developing kernels. As discussed previously, increased phosphorylation likely prevented overformation of dityrosine in the developing wheat kernels, therefore leading to a higher quality flour for bread-making.

Table 1. Level of dityrosine formed in wheat kernels harvested from plants grown with various phosphorous treatments

N:P:K level of fertilizer	Area under dityrosine peak
0:0:0 (control)	0.1792
10:15:10	0.0557
10:60:10	0.0530

Example 25

This example compares several wheat varieties grown under heat stress conditions and the effect of phosphorylation upon dityrosine formation. These samples are analyzed for dityrosine, tyrosine, and phosphotyrosine levels.

Materials and Methods

Wheat plants from three cultivars (varieties) of hard red winter wheat (varieties KARL 92, TAM 107, and Jagger), two cultivars of hard white winter wheat (varieties Betty and Heyne), and one variety of hard red spring wheat (Chinese Spring) are tested in this experiment. Samples of the hard red and hard white winter wheat varieties are germinated in vermiculite for 5-7 days, sprouted and subsequently vernalized for six weeks at 4°C in a growth chamber. The Chinese Spring variety is also germinated in vermiculite for 5-7 days until sprouting but is then immediately planted. The experimental plants are fertilized as described above in Example 24 and grown under greenhouse conditions of 25 °C during the day and 20 °C during the night. Thereafter, 2-3 pots of each cultivar are moved to a greenhouse unit set at 40 °C at the following intervals: 5 dpa, 10 dpa, 12 dpa, 15 dpa, 18 dpa, 20 dpa, 22 dpa, 25 dpa, and 30 dpa. Each of these pots is fertilized with the same N-P-K fertilizer used previously every two weeks during the time they are exposed to the 40 °C temperature. All

seed is then harvested at maturity and analyzed for the presence of dityrosine, tyrosine, and phosphotyrosine using methods previously described.

Results

The increased phosphorous levels in fertilizer at both standard fertilization intervals during the initial growth of the plants (prior to anthesis), or during the kernel maturation process (after anthesis) has a protective effect upon storage protein (glutenin) and ultimate bread-making quality. The increased levels of phosphorous cause increased phosphorylation of the glutenins and prevent the over formation of dityrosine in kernels under heat stress. Thus, when crops are under heat stress, dityrosine levels of the growing wheat can be measured and subsequently controlled by the application of phosphorous. This allows the crop producer to take an active role in protecting wheat quality during environmental stress conditions and consistently produce high quality wheats.

Example 26

This example compares dityrosine formation in soft wheats which are generally regarded as being of low quality for breadmaking. Soft wheats do not form good quality doughs but rather form batters due to their inability to form gluten.

Materials and Methods

Two soft wheat flours (5g of both Pioneer 2550 and Pioneer 2555) were thoroughly mixed together and a 50 milligram sample of the flour and a 10 minute mixed dough (10g) made from the soft wheat flour during standard mixograph analysis were examined for the presence of dityrosine using previously described methods. The flour sample and the dough sample made from the soft wheat flour were then compared to a standard analysis from hard wheat (NWS) control dough which had been mixed for 10 minutes.

Results

Fig. 35 shows the chromatograms from these analyses. The soft wheat flour has a very small amount of dityrosine and the dough mixed for 10 minutes made from the soft wheat flour has the same amount of dityrosine, indicating that there was no increase in the level of dityrosine during mixing as has been previously detected when mixing hard wheat (wheat suitable for bread-making) flour. The standard analysis from the NWS control dough is shown for comparison and exhibits a much higher peak. There is an increase in the second peak, which we have referred to as the "25" minute peak, during mixing of the soft wheat flour. However, this peak has not proven to be nearly as significant as the dityrosine peak.

Thus, dityrosine formation is integral to the formation of gluten. Hard wheat doughs form gluten and also show increased levels of dityrosine during all stages of the mixing and

baking processes. Soft wheats do not form gluten and instead of forming a dough, they form a batter. As evidenced by the similarity of the peaks from the soft wheat flour and the soft wheat dough, dityrosine does not form appreciably during the mixing of soft wheat flour. However, this is entirely expected in the formation of batters since batters are very different from doughs which need to have certain visceolastic properties that are helpful in bread-making. Soft wheats such as these are typically used for making products such as cookies, crackers, sponge cakes and udon noodles. These products do not require the development of a gluten structure such as that found in bread dough. In fact, studies have shown that the strength of gluten developed by a flour's protein is negatively associated with pastry quality, as measured by sugar-snap cookies. Souza, et al.; *Association of Sugar-Snap Cookies With High Molecular Weight Glutenin Alleles in Soft White Spring Wheats*, 71*Cereal Science*, 601-605 (1994). Pastry wheats require weak gluten strength for superior quality. High molecular weight glutenin alleles that cause development of strong glutenin have been shown to be detrimental to pastry quality. The glutenin strength, denominated as GRS score (glutenin rank sum) of cultivars and breeding lines has been shown to be negatively correlated to cookie diameter. The recommendation was that selection of cultivars with low GRS scores should provide cultivars with better and more predictable sugar snap cookie quality. Souza, et al. 1994. The GRS score is the summed effects of pooling the three loci in wheat that code for the glutenin subunits. Individual glutenin subunits did not have as much influence on cookie diameter as the total complement of glutenin subunits in a given wheat.

Example 27

This example compares dityrosine levels between Durum wheat flour and doughs made with Durum wheat flour.

Materials and Methods

A durum wheat flour and a 10 minute mix dough made from the durum wheat flour during standard mixograph analysis were examined for the presence of dityrosine using previously described methods.

Results

The results of this example are shown in Fig. 36. The durum wheat flour has a fairly high amount of dityrosine that has already formed in the kernel, however the dough made from the durum wheat flour and mixed for 10 minutes has approximately the same amount of dityrosine. This indicates that there was no appreciable increase in the level of dityrosine during mixing as has been previously detected when mixing bread wheat flour. This is in contrast to the normal increase in dityrosine levels seen during all stages of the mixing and baking processes of flours suited for bread-making. However, this is entirely expected because

durum wheats are typically used for making products such as pasta, although there are some durum wheat varieties which have been bred for their bread-making potential. Development of the gluten structure is not necessary for pasta products, although some researchers have argued that a small amount of gluten development may occur in pasta production during extrusion. Durum wheats differ from bread wheat most significantly due to the fact that they contain only 2/3 of the genetic material that is contained in bread wheat. Wheat used for bread-making contains three genomes, denominated "A," "B," "D," genomes, that were derived from three different wild grasses which were crossed resulting in the common bread wheat of today. Durum wheat is the result of only two of these grasses crossing and so it contains only genomes A and B rather than all three. Several of the proteins that form gluten are missing in durum wheat. The most notable of these are the high molecular weight glutenin subunits that are quoted for on the "D" genome, which is missing in these durum wheats.

Example 28

This example measured the effects of adding a common reducing agent/free radical scavenger (cysteine) to doughs before mixograph analysis. These mixograph analyses were performed with a standard good bread-making quality control flour.

Materials and Methods

Doughs incorporating five different levels of free cysteine were compared to a control dough and a cysteine standard after mixograph analyses. The cysteine levels included 40 ppm cysteine, 200 ppm cysteine, 0.5% cysteine, 2.5% cysteine, and 5.0% cysteine. After mixograph analysis, doughs were placed in liquid nitrogen immediately after mixing and then lyophilized to dryness. Dityrosine analyses on the doughs were then performed as described previously. Fig. 37 shows the comparative dityrosine analyses from each dough incorporating cysteine, a control dough, and the cysteine standard.

Results

As shown by Fig. 37, any incorporation of cysteine prevented the formation of dityrosine. The only dough exhibiting the presence of dityrosine was the control dough as shown at the peak at approximately 22 minutes. The peak which appears at approximately 16 minutes is likely a compound containing cysteine and tyrosine linked together. Thus, the presence of cysteine has a profound effect upon dityrosine formation in doughs. Therefore, the reducing agent/free radical scavenger cysteine, when incorporated into a dough system, prevents the ability of the dough to form dityrosine. Doughs containing cysteine do not form proper doughs and the mixograph analyses indicate very poor quality mixing properties. However, reducing agents/free radical scavenger such as cysteine are commonly used in the baking industry and are added to doughs when the doughs are too "bucky" or too elastic and

do not relax properly. In cases such as these, the incorporation of cysteine prevents further dityrosine formation and helps to "relax" the dough.

Example 29

This example compares the dityrosine analyses from dough samples which were mixed with varying levels of free glutathione. These samples were then compared to a dityrosine analysis from a control dough and the dityrosine analysis from a glutathione standard. Similar to Example 28, mixograph analyses were performed with a standard good bread-making quality control flour.

Materials and Methods

The reducing agent/free radical scavenger (glutathione) was added in two different levels to the above referenced doughs and compared to the glutathione standard as well as the control dough. Levels of glutathione included 100ppm and 500ppm glutathione. After mixograph analyses, doughs were placed in liquid nitrogen and then lyophilized to dryness. Again, dityrosine analysis on the doughs were performed as described previously. Results of this example are given in Fig. 38.

Results

As shown by Fig. 38, the presence of glutathione inhibits the formation of dityrosine. The effect of this inhibition is that dityrosine formation is nearly entirely prevented. However, there is a very small dityrosine peak in the glutathione containing examples, but it is extremely small compared to the control level. The dityrosine peak appears at approximately 22 minutes. Therefore, the reducing agent/free radical scavenger glutathione, when incorporated into a dough system, prevents the ability of the dough to form dityrosine. Doughs containing glutathione do not form proper doughs and the mixograph analyses indicate very poor quality mixing properties. However, reducing agents/free radical scavengers such as glutathione are commonly used in the baking industry and are added to doughs when the doughs are too "bucky" or too elastic and do not relax properly. In cases such as these, the incorporation of glutathione prevents further dityrosine formation and helps to "relax" the dough.

Example 30

This example sets forth methods for determining wheat protein (glutenin and gliadin) genes which are either up regulated or down regulated in response to heat exposure stress and/or differing phosphorous levels during wheat growth. As shown previously, genetically identical cultivars respond differently under these conditions, and this is correlated with the content of tyrosine, dityrosine and phosphotyrosine in the growing plants. Accordingly, knowledge of which genes are up regulated or down regulated permits control and

manipulation of the genes through conventional methods so as to produce optimum wheat plants which can be consistently grown in given environmental conditions. The method makes use of differential displays of messenger RNAs (mRNAs) in order to demonstrate relative degrees of up or down regulation.

Materials and Methods

Three groups of wheat plants from the same seed stock (of identical genotype) are grown under different temperature conditions and different fertilization levels using conventional N-P-K fertilizers. Group A is grown under greenhouse conditions at 20°C and the plants are fertilized at 2, 4, and 6 weeks after planting using either 10-60-10, 10-15-10, or 20-20-20 N-P-K fertilizer. Group B is grown under greenhouse conditions at 40°C and at the same three fertilizer concentrations. Group C is grown under normal environmental conditions and is fertilized using the same fertilizer procedure used for groups A and B. Wheat kernels are removed from the heads at 5, 10, 15, and 25 dpa and examined along with mature seed from the same batch of plants. Once the kernels are harvested, they are immediately immersed in liquid nitrogen and frozen at -80°C. Once the wheat kernels are removed, differential mRNA displays are used to identify and isolate genes that are differentially expressed between the various groups of wheat samples. Additionally, kernels from each wheat sample are analyzed for the presence and level of tyrosine, phosphotyrosine, and dityrosine using previously described methods. To identify the differentially displayed mRNAs, poly A mRNA for each sample is first purified by treating it with DNase to remove contaminating DNA. Next, the poly A mRNA is reverse transcribed to cDNA using reverse transcriptase and oligo dT12-18 anchor primers which contain additional bases at the 3' end. The cDNA is then amplified using PCR with arbitrary primers and anchor primers used for reverse transcriptase. The resulting products are then subjected to electrophoresis side by side on polyacrymalamide gel. As the samples are run in parallel, the procedure allows for the simultaneous detection of differentially expressed genes. Additionally, gene expression over a time continuum can also be measured using this method. Results from this electrophoresis are then compared and differentially expressed cDNAs are identified. Once these cDNAs of interest are identified, the bands of interest are excised. Next, the excised DNA is reamplified using primers from the primary reaction. Reamplified DNA is then reanalyzed on agarose gel and differential expression is confirmed using northern blot of RNA from the original sample populations using the labeled product DNA as a probe. Finally, the cDNAs of interest are cloned and sequenced using conventional techniques.

Results

Using these techniques, genes which are either up regulated or down regulated under varying stress conditions can be readily identified. With this information, transgenic wheats

can be produced using known methods in order to optimize the response of the wheat to the known stress conditions. Of course, this same technique can be employed with other plants to achieve the same ends. response to exposure to heat stress and/or differing phosphorous levels. Thus, genes which are either up regulated or down regulated in response to these environmental stresses can be controlled and manipulated such that optimum wheat plants can be consistently grown despite environmental conditions. Additionally, correlations between the levels of tyrosine, phosphotyrosine, dityrosine, and the environments and fertilizer regimes to which the plants were exposed demonstrates the protective effect that phosphotyrosine has on developing wheat kernels.

Example 31

In this example, various peptides each containing a tyrosine pair (YY) were tested to determine their effectiveness in forming dityrosine linkages. Specifically, the different sequences were tested to determine their ability to form dityrosine linkages with high molecular weight glutenins.

Materials and Methods

Four peptides of varying lengths were synthesized using conventional methods at the Kansas State University Core Biochemical Facility. The peptide sequences are as follows: QQGYYPTS, QGYYPTS, YYPTS, and YY (dipeptide, not dityrosine). Y (free tyrosine) is used in all tests as a control. These four peptides were exposed to the following experimental conditions based or the work by Michon, et al., *Biochemistry* **36**:8504-8513 (1997). In this paper, tyrosine-containing peptides and derivatives were oxidized by horseradish peroxidase and dityrosine formation was investigated. The test peptides and derivatives used by Michon included the following: NAT (N-acetyltyrosine), NATA (N-acetyltyrosine amide), PQQPY, YPQQPA, PQQPYPQQPA, and QQMQQSPRSTRPYQQRPGQQ.

For this test, 0.5mM of each test peptide or derivative was reacted under each of the following conditions 1-3. The reactions were carried out in water, except for condition 2 and the temperature of each reaction is 37°C, except as noted in the case of $KBrO_3$. The reaction volume in each case is 1.0 ml.

1. Water control (24 hr reaction).

2. Water soluble extract from flour (NWS control flour) (24 hr reaction). The water soluble extract is made by adding water to flour in a 5:1 ratio and mixing in a beaker on a stirring plate for 1 hr. The flour/water mix is then centrifuged and the supernatant is removed and used as the "water soluble extract."

3. 30 ppm $KBrO_3$ at 200°C for 25 minutes (this 25 minute reaction, mimics baking conditions).

The peptides and derivatives were allowed to react for the specified times under each of the foregoing experimental conditions. The respective reaction solutions were then lyophilized to dryness and amino acid hydrolysis is performed according to methods previously described. Dityrosine contents were analyzed by HPLC amino acid analysis, also as previously described.

The water-soluble extract from flour is used because cereal chemists have reported that the water-soluble extract of flour contains some unknown compound that causes dough breakdown during mixing. Shroeder, et al., *Mixograph Studies II Effect of Activated Double Bond Compounds on Dough Mixing Properties*, Cereal Chemistry, **55**:348 (1978). Such breakdown has been associated with increased formation of dityrosine bonds (Example 1).

Finally, to determine whether the $KBrO_3$ was facilitating the formation of dityrosine through the formation of free radicals, as opposed to some other agent present in the solution, dityrosine formation in two solutions was compared. The peptide QQGYYPTS was added to one solution containing 30 ppm $KBrO_3$ as well as to another solution containing 30 ppm $KBrO_3$ and 500 ppm butylated hydroxytoluene (BHT) which is a common free radical scavenger. Each of these solutions were then tested for dityrosine formation using methods previously described.

This $KBrO_3$ experiment was done to determine if it is possible for these peptides or derivatives to form dityrosine bonds with only $KBrO_3$ in the buffer solution, at normal baking temperature and time. The highest amount of dityrosine bonds obtained under dough baking conditions occurred with $KBrO_3$-supplemented doughs (Example 15). Thus, this experiment assisted in the determination that $KBrO_3$ is the primary cause of dityrosine bond formation (by oxidation/free radical scavenging), and that there is not some other agent in the dough and bread required for dityrosine bond formation.

Results

Results of this example are given below in Table 2.

Table 2. Level of dityrosine formed among peptides under various conditions

Peptide sequence or control	Area under dityrosine peak (in $KBrO_3$ + 200°C	Area under dityrosine peak (in water sol. extract of flour)	Area under dityrosine peak (in water only)
QQGYYPTS	13.76	9.87	0
QGYYPTS	26.29	2.68	--
YYPTS	3.86	2.03	--
YY	42.06	1.72	--
Free Y	5.9	1.24	--
QQGYYPTS + 500 ppm BHT	4.47	--	--
Water soluble extract of flour (control)	--	0.0506	--
BHT control (+200°C)	--	--	0
Bromate control	0	--	--

All of the peptides were able to form dityrosine when incubated in 30 ppm aqueous $KBrO_3$ at 200°C for 25 minutes. In this experiment, the dipeptide (YY) was the most efficient at forming dityrosine followed by QGYYPTS, QQGYYPTS and YYPTS. The control peptide QQGYYPTS did not form dityrosine when incubated in water at 200°C for 25 minutes. Thus, the $KBrO_3$ causes dityrosine formation even without any other factors which might be present in a dough or bread. If there were an agent in the flour that is required for dityrosine formation, dityrosine would not have formed in this experiment. Additionally, $KBrO_3$ was shown to significantly enhance dityrosine formation under these conditions because the control peptide formed no dityrosine when incubated under the same conditions but without the addition of $KBrO_3$.

When one of the solutions contained BHT in addition to $KBrO_3$ dityrosine formation was inhibited. Thus dityrosine formation was shown to be related free radical formation (or some other free radical mechanism) because the addition of BHT inhibited this formation.

Example 32

This example describes site directed mutagenesis of DNA coding for QQGYYPTS repeats and variations thereof in expressed glutenin proteins in order to analyze the effect of the precise repeat amino acid sequence on dityrosine formation in doughs.

As demonstrated above, dityrosine crosslinks form between and among glutenin proteins during dough mixing, and the ability to form dityrosine bonds effects product quality. Accordingly, controlling the ability of glutenin proteins to form dityrosine bonds grants the ability to control ultimate product quality. In order to determine the significance of the QQGYYPTS (glutamine-glutamine-glycine-tyrosine-tyrosine-proline-threonine-serine) repeats (which periodically occur in the amino acid sequences of the glutenin subunits) on dityrosine formation between and among the high molecular weight glutenin subunits (HMW-GS), various mutations of this repeat sequence in HMW-GS are made. Those proteins are expressed in *E. coli*, purified, and added into a base flour to test the effects of the mutations on product quality. Various quality analyses, including mixograph analysis and test baking, are performed in order to assess the precise structural contributions of the amino acids of the repeat sequence. These proteins are also expressed in transformed wheat plants. The quality of the resulting flours are tested for their mixing and baking properties.

Materials and Methods

1. Isolation of the high molecular weight glutenin gene.

High molecular weight glutenin subunit 1Dx5 DNA. This subunit is selected because it is known to be associated with good breadmaking quality wheat. It is to be understood that other glutenin subunits could also be isolated and treated as described below.

Plasmids containing the coding region of Glu-1-Dx5 and other HMW-GS have been reported in the literature (Sugiyama et al (1985), Anderson et al. (1989), Galili (1989), and Halford et al (1992)). If such plasmids are not available, the gene encoding subunit Glu-1-Dx5 may be isolated from genomic/cDNA libraries.

Genomic and cDNA libraries can be constructed or prepared by a suitable biotechnology company such as Stratagene. For commercial procedures, tissue or purified genomic DNA or mRNA can be obtained. The library(ies) are screened using a DIG labeled probe, e.g., a clone of PCR amplified repeat segment of the HMW-GS Dx gene. Following several rounds of screening/amplification of positive clones, *in vivo* excision of phage DNA to a phagemid (pBluescript SK+) is performed as described in the manual supplied with the library (Lambda Zap II library instruction manual, Rev. #127001c.

The plasmids are prepared by the alkaline lysis method using a Qiagen miniprep kit (Valencia, CA), and the correct sequence is identified by restriction analysis and partial dideoxy DNA sequencing.

2. Generation of sequences modified in number, location and precise amino acid sequence contained in the "QQGYYPTS" repeats of the HMW glutenin subunit.

a. Site-directed mutagenesis.

Synthetic oligonucleotides incorporating the desired sites (5'-caacaaggttactacccaacttct-3' codes for: QQGYYPTS) and also containing the appropriate flanking sequences are synthesized and phosphorylated at the 5' end. The flanking sequences can be 10-30 bp in size and can therefore increase the specificity of the insertion. For any given site in the glutenin protein sequence, an insertion of the (5'-caacaaggttactacccaacttct-3') sequence can be made if the primer used during PCR amplification also contains flanking regions that are specific for up to 22 bp on either side of the desired insertion location.

Oligonucleotides with the following variations on the QQGYYPTS sequence are made: The actual mutated bases are underlined and shown in bold

	QQGYYPTS	5'-caacaaggttactacccaacttct-3' (Wild type)
1.	QQGFFPTS	5'-caacaaggtttcttcccaacttct-3'
2.	QQGYFPTS	5'-caacaaggttacttcccaacttct-3'
3.	QQGFYPTS	5'-caacaaggtttctacccaacttct-3'
4.	QQGAAPTS	5'-caacaaggtgccgccccaacttct-3'
5.	QQGYAPTS	5'-caacaaggttacgccccaacttct-3'
6.	QQGAYPTS	5'-caacaaggtgcctacccaacttct-3'
7.	QQGCCPTS	5'-caacaaggttgctgcccaacttct-3'
8.	QQGYCPTS	5'-caacaaggttactgcccaacttct-3'
9.	QQGCYPTS	5'-caacaaggttgctacccaacttct-3'

Although these sequences are based upon the sequences prevalent within the HMW-glutenin genes of *Triticum spp.*, and codons derived from the latest codon usage of *T. aestivum* genes, other codons that code for the amino acids may work equally well or better.

Codon usage tabulated from the international DNA sequence databases; its status 1999. Nakamura, et al., *Nucleic Acids Research.* 27:292 (1999).

The protocols for site directed mutagenesis are based upon those published by Deng and Nickoloff (1992) and Lewis and Thompson (1990), and have been simplified by the commercialization of products such as Stratagene Chameleon™ Double-stranded site directed mutagenesis kit, and Promega Altered Sites® II in vitro mutagenesis system. These products allow double stranded plasmid DNA to be used for site-directed mutagenesis using selection

by incorporation of a unique restriction site or antibiotic resistance in the altered strand of the plasmid. Generally, the mutagenesis protocol involves the following steps:

1. Subclone the DNA to be mutagenized into high-copy-number vector (pBluescript SK+) or vector specific for kit.

2. Prepare template DNA by plasmid miniprep. Resuspend 100 ng of the DNA in TE buffer at a final concentration of 1 ng/µl.

3. Synthesize oligonucleotide primer. The 5' and 3' ends of the primer consist of the "clamp" sequences (homologous to the template DNA) and in between is the sequence to be inserted or mutated (5'-tactacccaacttct-3' or variations thereof). Other primers are synthesized for other regions of the sequence. This section describes only the synthesis of areas of the DNA containing the insertion sites. Primers for mutagenesis must be 5' phosphorylated. This may be chemically performed during synthesis or added to a primer using T4 polynucleotide kinase and ATP as phosphate donor.

4. Purify synthesized oligonucleotides by denaturing polyacrylamide gel electrophoresis. Recover oligonucleotides and resuspend in TE buffer. Determine absorbance at A280 and adjust the concentration to 500 ng/µl (100pmol/µl), if necessary. Another option is to purchase high quality oligonucleotides.

5. If pBSK+ is utilized, primers for selection will transform the KpnI site in the plasmid polylinker to an SrfI site. Other plasmid vectors may involve change of antibiotic resistance.

6. In a single 1.5 ml microfuge tube add 0.25 pmol plasmid template, 25 pmol each of selection and mutagenic primers, 10x mutagenesis buffer and appropriate amount of ddH2O. Boil the tube in a water bath for 5 min and then place on ice for 5 min. Incubate for 30 minutes at room temperature. This step denatures (separates) the two DNA strands of the plasmid, and allows the primers to anneal to complementary regions. This method may be modified using alkaline denaturation and controlled cooling to better control annealing of oligonucleotide primers, and prevent the double stranded plasmid from re-annealling. A thermal cycler is very useful to have a programmable rate of cooling to optimize primer annealing. Note that primers for selection and mutagenesis must not overlap and must be complementary to sequences on the same DNA strand.

7. A mixture of the enzymes DNA polymerase and DNA ligase are added to synthesize mutant strand DNA and ligate the newly synthesized strands. Prior to adding the enzymes a mix of deoxy nucleotide triphosphates (dNTPs) are added. The mix is incubated at 37°C for 60-90 minutes.

8. If selection involves change of a restriction site, digest plasmids with the restriction enzyme that recognizes the original site. This will generate linear DNA in the unmodified plasmid.

9. Transform *E. coli* cells bearing a mutS genotype (repair minus strain) with the plasmid. These cells are deficient in mutation repair mechanisms and thus will not "correct" the introduced mutation. Linear DNA transforms to a much greater efficiency than circular/supercoiled plasmid DNA.

10. Plate cells on solid media containing appropriate antibiotic for selection. (This may be the selection step if antibiotic resistance is used as the selection mechanism). Media may contain X-gal and IPTG for blue/white selection to verify Lac phenotype.

11. Analyze colonies that display positive selection by growing overnight cultures, purification of plasmids and analysis by restriction profiles (e.g., insert size as well as presence of new restriction sites generated by mutagenesis).

12. Transform competent cells (e.g. XL-1 Blue strain) with plasmids that have passed first selection criteria.

13. Grow colonies of interest and analyze. Ultimately DNA sequencing is required to verify desired mutations. A similar process as the one described above can also be used to remove an already existing QQGYYPTS site in a protein. However, the inserted sequence (flanked by homologous 5' and 3' ends) would be altered to include the alternate sequence desired. In this case also, the primer ends would be homologous to the regions of the protein just before and after the already existing 5'-caacaaggttactacccaacttct-3' sequence in the template. That portion of the template would not be amplified in that case. The resulting expressed protein would be missing that particular QQGYYPTS site.

3. Purification of the HMW-GS with variations in the QQGYYPTS sites and incorporation into flour/dough analyses:

a. Transformed *E. coli* is grown on 2YT medium (0.5L in 1.5L flasks) at 37°C until the OD_{600} is between 0.6 and 0.8 and is then induced by the addition of isopropyl-b-D-thiogalactoside to a final concentration of 0.4mM. The bacterial cells are harvested 18 h after induction. Pelleted cells are dissolved in 70% (v/v) DTT (dithriothreitol) and the peptides are extracted at 60°C for 2 h. After centrifugation for 15 min. at 13,000g, ethanol is added to the supernatant to a final concentration of 90% (v/v). The resultant precipitate, collected by centrifugation is dissolved in 0.05M Tris-HCL buffer, pH 8.0, containing 4 M urea, dialyzed against water for 48 h and then freeze dried.

b. The freeze dried protein is then dissolved in 0.05 M glycine-acetate buffer, pH 4.6, containing 4 M urea and applied to a 10x2.5 cm column of carboxymethylcellulose (CMC) (Sigma, fast flow) equilibrated in the same buffer and eluted with a gradient of o-0.15M NaCl. The column effluent is monitored by OD_{280} and SDS-PAGE and fractions containing HMW subunit peptides dialyzed and lyophilized. Finally, fractions for N-terminal sequencing are separated by reversed phase (RP)-HPLC. The freeze-dried extracts are dissolved at a concentration of 3 mg/mL applied to a Vydac C_8 semi-preparative column (10 mm X 25 cm) at 50°C using a System Gold (Beckman) HPLC with a model 126

solvent delivery system and a model 166 detector. Elution is with a linear gradient of water containing 0.07% (v/v) trifluoroacetic acid (TFA) (A) and acetonitrile containing 0.05% (v/v) TFA (B), from 18% B to 23% B over a 40 min period at a flow rate of 2.5 ml/min. The column effluent is monitored at 210 nm and fractions containing the HMW subunit peptides are lyophilyzed. For the preparation of alkylated peptides, an excess of 4-vinylpyridine os added to the dissolving buffer and the reaction allowed to proceed for 10 min at 60°C prior to application to the column. N-terminal sequencing is performed on the peptides alkylated with the 4-vinylpyridine, using a pulsed-liquid amino acid sequencer (model 477A, Applied Biosystems) equipped with and online phenylthiohydantoin-amino acid analyzer (Model 120A).

c. Purified peptides are re-oxidized *in vitro* to form disulfide crosslinks using potassium iodate. Re-oxidized samples are separated by SDS-PAGE using a multi-stacking system to check purity and identity.

d. Mixing studies are conducted in triplicate with a 2-g Mixograph using a modification of the standard method for 35 g of flour scaled to the two gram size. Mixing parameters are determined using a modification of a previously reported MixSmart computer program. This modification automatically excises the portions of the recording during which mixing is halted. Multiple analysis of variance followed by the student-*t*-test is used to compare mixing parameters from different treatments using the MSUSTAT statistical program package. Least significant differences (L.S.D.) are determined at the 5% probability level.

e. A reversible reduction/oxidation procedure for incorporating added peptides (5 mg) into glutenin is used. A special flour with 16.2% protein content milled from a *Glu-D1* null line with a mixed Gabo/Olympic background, containing subunits 1Ax1, 1Bx17 and 1By18, is used as a base flour. Flour is mixed with 1.0 mL water and 0.1 mL water containing 324 mM DTT for 30 s and allowed to react for 4 min. The reduced doughs are then treated with 0.1 mL oxidant solution containing 934 mM of potassium iodate. Mixing is continued for 30 s and the dough is then allowed to react for 5 min and then mixed for a further 10 min, as in a conventional Mixograph determination.

f. Expressed protein (250 mg) is also mixed into 100 gm of the base flour and baked into bread using the AACC 10-10B breadmaking procedure. Bread quality parameters are evaluated and compared in order to assess the effects of adding HMW-GS with the altered repeat sequences.

g. During the mixing and baking studies, samples of dough and bread are taken at various stages of processing and analyzed for the presence of dityrosine by procedures previously described. The expressed proteins which allow the highest level of dityrosine formation during mixing and baking studies are those that possess the sequence that is most conducive to dityrosine formation. This procedure allows testing of variations on the

QQGYYPTS sequence within the glutenin subunits and determination of the importance thereof in the formation of dityrosine during mixing and baking.

4. Expression of the HMW-GS in wheat plants (plant transformation).

Plots of wheat (*Triticum aestivum* L.) are grown at regular intervals, in 6 inch diameter plastic pots filled with Ready Earth (W.R. Grace & Co.) potting mixture, to maintain a steady supply of plant material. Plants are maintained in a growth chamber at 25± 2°C and 20± 2°C night temperatures under a 16 h photoperiod (150 µmol photon m^{-2} sec^{-1}) provided by banks of fluorescent tubes and incandescent bulbs. All plants are watered every second day and fertilized once a week with 0.4 g/l of soluble greenhouse fertilizer (20:20:20).

a. Preparation of scutella: Wheat spikes are harvested 10-12 days post anthesis from greenhouse grown plants. Immature caryopses are removed from the spikelets and surface sterilized with 70% ethanol for 1 min and 20% (v/v) Javex bleach (1.2% sodium hypochlorite) for 20 minutes followed by five rinses with sterile double distilled water. Using a stereo dissecting microscope, the immature embryos are aseptically removed from the caryopses and the scutella isolated by carefully removing the embryo axis. The isolated scutella are then cultured with their abaxial (convex) surfaces in contact with the medium. The culture medium should consist of MS (Murashige and Skoog, 1962) medium supplemented with 2 mg l^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D) and 100 mg l^{-1} casamino acid, and herein after referred to as MS^{++} medium. The medium is solidified with agar (0.8%) and the pH adjusted to 5.8 prior to autoclaving. The cultures are incubated in the dark at 26 ± 2°C for 1 week and then transferred to low light (10 µmol photons $m^{-2}sec^{-1}$) under a 16 h photoperiod for another 2-3 weeks.

b. DNA coating and bombardment: Gold particles (1.0 µm) are coated with plasmid DNA using a procedure modified by Klein*et al.* 1988). Prewashed 50 µl aliquots of gold particles (60 mg particles suspended in 1 mL deionized water) are mixed with 10 µL of plasmid DNA (1 mg ml^{-1}), 50 µl $CaCl_2$ (2.5M) and 20 µl spermine (0.1 M) in a microfuge (1.5 ml) tube by vortexing after each addition. The mixture is vortexed for 3 min at room temperature and centrifuged in a microfuge for 20 sec. The supernatant is removed and discarded. The DNA coated particles are then washed again with 250 µl of ethanol and resuspended in 60 µl of ethanol. The suspension is pipetted out in 10 µl aliquots on to microcarriers for bombardment.

The isolated scutella is be precultured for 2 days on MS^{++} medium in the dark prior to bombardment. Twenty precultured scutella are arranged in a circle of about 2 cm diameter in the center of a 60 x 15 mm petri plates containing 10 ml of agar solidified MS^{++} medium. The petri plates are positioned at 115 mm target distance in such a way that the abaxial (concave) surface of each scutella was in the direct path of the micro projectiles. The microprojectiles are bombarded at a rupture pressure of 1100psi using the DuPont helium driven Biolistics particle delivery system (PDS 1000).

c. Selection of transformants: Following bombardment, the scutella are cultured in the dark for 2 days on callus induction media containing 0.4 M mannitol for 4 h before and 20 h after bombardment. Bialaphos resistant embryos are selected on half MS media supplemented with 1 mg/L of bialaphos (Meiji Seika Kasha, Tokyo, Japan). The somatic embryos formed on this media are separated and to MS^{++} media. The embryos capable of developing into green shoots within 3-4 weeks are characterized as putative transformants. The selected shoots are transferred to half MS for further growth and rooting. The plantlets developed on this media are transferred to environmentally controlled growth chambers and grown for further analyses.

Protein is extracted from immature endosperm tissue and screening for the presence of the HMW-GS 1Dx5 will be performed by SDS-PAGE analysis.

Plants found to contain the modified HMW-GS are used to increase the amount of seed available by subsequent planting of seed from these plants. Once enough seed is available, all standard analytical tests as well as milling, mixograph and baking analyses are performed to assess the quality of the grain developed with the various modifications of the QQGYYPTS repeats. Dough and bread made from these kernels are also evaluated for the presence of dityrosine. A comparison of the levels of dityrosine present in the flour, mixed dough and baked bread, as well as the various quality parameters of these products, are used to assess the effects of modifying the YYPTS sequence on product processing quality.

5. Expression of the storage proteins in plants other than wheat.

Any protein can be modified in transformed plants by the aforementioned techniques. Therefore, if modification of barley proteins, soybean proteins or any other protein is desirable, the above procedures can be performed using the sequence from the protein selected for modification as the template and simply inserting the 5'-caacaaggttactacccaactct-3' sequence or modifications thereof wherever the sequence is desired.

Example 33

This example shows that common wheat substitutes, often used by people who cannot eat wheat, do not form significant levels of dityrosine during processing. This lack of dityrosine formation accounts for their production of inferior products because the substitute ingredients do not have the same properties as wheat flour and hence cannot form good quality doughs. Accordingly, dityrosine cross linking capabilities could be edited into these grains and their potential for wheat replacement in various products would be greatly enhanced.

Materials and Methods

Mixograph analyses were performed using the standard mixograph procedure previously described and testing doughs formed with flours of the following grains: barley, oat, amaranth, rye, buckwheat, rice, millet, kamut, soy, and triticale. The doughs comprised a

mixture of flour from those grains and water only. Dityrosine analyses were performed on the flours and on doughs made from these flours which had been mixed for ten minutes. The dityrosine analyses were performed using the procedures previously described.

Results

None of these flours formed viscoelastic doughs but rather the mixture resembled a batter. This is because wheat gluten has properties of viscoelasticity and particularly good binding properties that no other grain can mimic sufficiently to form viscoelastic doughs. In fact, none of the wheat substitutes tested exhibited a significant dityrosine levels including triticale (which is a cross between rye and wheat), or kamut (which is a relative of wheat).

Discussion

Monitoring and/or measuring tyrosine bond formation may now be used to consistently produce optimum dough products. Standards for tyrosine bond content corresponding to different dough applications and different processing stages may now be found and used as a comparison or guide to direct dough processing. Moreover, the starting tyrosine and dityrosine content in flour may also be found and used to both predict dough characteristics and precalibrate dough processing equipment such that waste is minimized and optimum products are consistently produced. Knowledge of this starting tyrosine and/or tyrosine bond content allows for prediction of the potential for tyrosine bond formation since the tyrosine present in the flour is the tyrosine used to form tyrosine bonds. A sample of flour exhibiting high levels of tyrosine and/or low levels of dityrosine should have the potential to form a higher number of tyrosine bonds. This in turn may contribute to a higher rate of tyrosine bond formation and therefore, shorter mixing times for dough formation. Moreover, a high starting tyrosine content may also indicate the need for modification such as increasing the pH of the dough or the addition of metal chelating agents or free tyrosine to retard tyrosine bond formation. Conversely, a flour exhibiting a lower level of tyrosine or higher level of dityrosine may need longer mixing times to form a high quality dough and/or some modification to promote tyrosine bond formation (i.e. the addition of oxidizing agents or decreasing the pH of the dough). The tyrosine and dityrosine content of flour may be measured through derivatized or underivatized amino acids, however, measuring the derivatized amino acids is a much more sensitive technique. An example of determining the tyrosine content of flour using HPLC of the underivatized amino acids is shown in Fig. 16. Tyrosine is represented by the peak which elutes at 12.380 minutes.

Additionally, environmental conditions were shown to have an effect on dityrosine content of flour and these conditions effected different wheat varieties to different extents. Thus, knowledge of environmental conditions will contribute to knowledge of what properties doughs made from such flours will exhibit. Moreover, varieties which are better suited for

particular environments may be selected for growth in these environments. Alternatively, the amino acid profiles of lower quality varieties can be genetically manipulated by cross-breeding or DNA recombination/alteration in order to produce new higher quality varieties. Another alternative would be to alter the environmental conditions (such as adding phosphorous or phosphorous-containing fertilizers to the soil surrounding growing wheat) in order to inhibit premature dityrosine formation.

Conclusion

Because tyrosine bond levels and the formation of tyrosine bonds were heretofore unrelated to dough formation capabilities and since the content of tyrosine and tyrosine bonds as well as tyrosine bond formation may be assessed both prior to and during dough production, dough production can be effected such that optimum dough products are consistently produced based on the knowledge of the tyrosine, dityrosine, phosphotyrosine and/or tyrosine bond content of the starting flour, assessment of tyrosine bonding during dough production and modification of tyrosine bond formation during dough production in response to the assessment in order to achieve an ideal tyrosine bond content based on the eventual end use of the dough produced. Additionally, levels of the same compounds can be monitored during growth of wheat plants and the resulting wheat kernels can be graded according to such levels.

Furthermore, developing wheat plants can be analyzed to determine their levels of tyrosine, dityrosine and phosphotyrosine and knowledge of these levels in the developing plants contributes to methods of altering such levels in order to produce wheat plants having optimum characteristics. This alteration can be done in a variety of ways including applying phosphorous to the ground adjacent growing plants and altering the genome of the plant such that the number of base pair sequence subunits coding for preferred peptide sequences are either increased or decreased. Accordingly, similar alterations can also be performed in non-wheat plants in order to produce products having crosslinking characteristics similar to products produced by conventional wheat doughs.

Finally, because of tyrosine bonding, polymers and composite polymers (synthetic and biopolymers) can be modified such that the peptide sequences described above can be coupled with the polymers in order to effect crosslinking between and among polymer subunits. In the case of biopolymers, the peptides can be directly incorporated into the normal protein sequence or attached as a side chain or end cap to the protein. Crosslinking reaction conditions are dependent upon the specific polymers present, however, the rate of crosslinking can be affected by the addition of oxidizing agents, free radical scavengers, reducing agents, and free radical generators in a similar fashion to that found in dough processes.

References

Anderson, O. D., Greene, F. C., Yip, R. E., Halford, N. G., Shewry, P. R., and Malpica-Romero, J. -M. 1989. Nucleotide sequences of the two high-molecular-weight-glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyenne. *Nucleic Acids Res.* **17**:3156-3157.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) Enzymatic manipulation of DNA and RNA. John Wiley & Sons NY, NY (Current Protocols in *Molecular Biology*, Vol I, pp 8.0.1-8.5.

Bekes, F., Gras, P. W., and Gupta, R. B. 1994. Mixing properties as a measure of reversible reduction and oxidation of doughs. *Cereal Chemistry* **71**:44-50.

Buonocore, F., Bertini, C. Ronchi, C. Bekes, F.Caporale, C. Lafiandra, D., Gras, P., Tatham, A. Greenfield, J. Halford, N. G., and Shewry, P. R. 1998. Expression and functional analysis of M_r 58000 peptides derived from the repetitive domain of high molecular weight glutenin subunit 1Dx5. *Journal of Cereal Science* **27**: 209-215.

Deng, W.P., and Nickoloff, J.A. 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Analytical Biochemistry* 200:81-88.

D'Ovidio, R., Anderson, O. D., Masci, S., Skerritt, J. and Porceddu, E. 1997. Construction of novel high-M_r glutenin subunit gene variability: Modification of the repetitive domain and expression in *E. coli*. *Journal of Cereal Science* **25**:1-8.

Galili, G. 1989. Heterologous expression of a wheat high molecular weight glutenin gene in *Escherichia coli*. Proceedings of the National Academy of Sciences (USA) 86:7756-7760.

Halford, N.G., Field, J.M., Blair, H., Urwin, P., Moore, K., Robert, L., Thompson, R., Flavell, R.B., Tatham, A.S., and Shewry, P.R. 1992. Analysis of HMW glutenin subunits encoded by chromosome A of bread wheat (*Triticum aestivum* L.) indicates quantitative effects on grain quality. *Theoretical and Applied Genetics* 83:373-378.

Klein, T. M., Gradzeil, T., Fromm, M.E. and Sanford, J. C. 1988. Factors influencing gene delivery into *Zea Mays* cells by high- velocity microprojectiles. *Bio/Technology* **6**: 559-563.

Lewis, M.K., and Thompson, D.V. 1990. Efficient site directed *in vitro* mutagenesis using ampicillin selection. *Nucleic Acids Research* 18:3439-3443.

Shani, N., Steffen-Campbell, J. D. Anderson, O. D., Greene, F. C., and Galili, G. 1992. Role of amino and carboxy-terminal regions in the folding and oligomerization of wheat high molecular weight glutenin subunits. *Plant Physiol.* **98**:433-441.

Sugiyama, T., Rafalski, A., Peterson, D., and Söll, D. 1985. A wheat glutenin subunit gene reveals a highly repeated structure. *Nucleic Acids Research* 13:8729-8737

Mutagenesis of Cloned DNA. pp 8.0.1-8.5.10 *In:* Current Protocols on Molecular Biology. 1997. John Wiley & Sons, Inc.

All references cited herein are specifically incorporated by reference, including all methods, standards, manuals, patents, publications and scientific papers.

I claim:

1. An isolated peptide selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14.

2. The peptide of claim 1, said peptide selected from the group consisting of YY, QQGYYPTS, QPGYYPTS, and reversals thereof.

3. An isolated peptide selected from the group consisting of peptides having the sequence QQGYYPTS and STPYYGQQ.

4. A composite polymeric structure comprising a pair of discrete biopolymers coupled together through a tyrosine bond, said tyrosine bond formed by two peptides respectively associated with each of said discrete biopolymers, at least one of said peptides being non-naturally occurring with respect to the corresponding biopolymer, each of said peptides being individually selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14.

5. The polymeric structure of claim 4, said biopolymers being selected from the group consisting of plant proteins.

6. The polymeric structure of claim 5, said biopolymers being plant proteins selected from the group consisting of wheat, soy, corn, rye, oats, triticale, sorghum, rice, and barley.

7. The polymeric structure of claim 5, at least one of said peptides being within and forming a part of the sequence of the corresponding plant protein.

8. The polymeric structure of claim 5, at least one of said peptides being attached as a side chain to the corresponding plant protein.

9. The polymeric structure of claim 4, there being a plurality of dityrosine bonds coupling said discrete biopolymers.

10. The polymeric structure of claim 9, said dityrosine bond being selected from the group consisting of dityrosine, isodityrosine, trityrosine, di-isodityrosine, and analogs thereof.

11. A polymeric structure comprising a biopolymer having respective portions thereof coupled together through a tyrosine bond, said tyrosine bond formed by two peptides respectively associated with said portions, at least one of said peptides being non-naturally occurring with respect to the biopolymer, each of said peptides individually selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14.

12. The polymeric structure of claim 11, said biopolymer being selected from the group consisting of enzymes and plant storage proteins.

13. The polymeric structure of claim 12, at least one of said peptides being within and forming a part of the sequence of the corresponding plant protein.

14. The polymeric structure of claim 12, at least one of said peptides being attached as a side chain to the corresponding plant protein.

15. The polymeric structure of claim 11, there being a plurality of dityrosine bonds coupling said portions.

16. The polymeric structure of claim 15, said dityrosine bond being selected from the group consisting of dityrosine, isodityrosine, trityrosine, di-isodityrosine, and analogs thereof.

17. A composite polymeric structure comprising at least a pair of discrete synthetic polymers coupled together through a tyrosine bond, said tyrosine bond formed by two peptides respectively associated with each of said discrete synthetic polymers, each of said peptides individually selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14.

18. The polymeric structure of claim 17, said synthetic polymers being selected from the group consisting of C_2-C_4 polyalkylene glycols and aminated and carboxy-capped derivatives thereof, polysaccharides and their carboxylated and aminated derivatives, the polyacrylates and derivatives thereof, the derivatized polyolefins, and the derivatized polystyrenes.

19. The polymeric structure of claim 18, at least one of said peptides being attached as a side chain to the corresponding synthetic polymer.

20. The polymeric structure of claim 17, there being a plurality of dityrosine bonds coupling said discrete synthetic polymers.

21. The polymeric structure of claim 20, said dityrosine bond being selected from the group consisting of dityrosine, isodityrosine, trityrosine, di-isodityrosine, and analogs thereof.

22. A method of altering a crosslinking property of a protein comprising the step of genetically altering a gene which expresses the protein in order to cause the altered gene to express a greater or lesser number of tyrosine bond-forming subunits in the protein.

23. The method of claim 22, said crosslinking property being an intraprotein crosslinking property.

24. The method of claim 22, said crosslinking property being an interprotein crosslinking property.

25. The method of claim 22, said protein being a plant protein.

26. The method of claim 25, said plant protein being selected from the group consisting of wheat, soy, corn, rye, oats, triticale, sorghum, rice, and barley.

27. The method of claim 22, said gene being altered to increase or decrease the number of base pair sequence subunits therein which code for YY.

28. A method for determining the approximate amount of tyrosine bonds in a dough comprising the steps of:

combining dough-forming ingredients to form a dough and mixing the dough; and

during said mixing step, analyzing said dough to determine an approximate range of tyrosine bonds within the dough.

29. The method of claim 28, wherein said analysis includes fluorometry.

30. The method of claim 28, wherein said tyrosine bonds comprise bonds between and among tyrosine residues and between tyrosine residues and a compound selected from a group comprising free tyrosine, quinones, hydroquinone, dihydroxyphenylalanine (DOPA), dopaquinone, semiquinones, glutathione (GSH), cysteine, catechols, various carbohydrates and analogs and combinations thereof.

31. The method of claim 30, said tyrosine bonds comprising dityrosine bonds.

32. A method of making a dough comprising the steps of:

combining dough-forming ingredients to form a dough and mixing the dough;

during said mixing step, periodically analyzing the dough to determine respective approximate ranges of the number of tyrosine bonds therein; and

comparing said analyzed ranges of tyrosine bonds to a predetermined optimum range standard in order to achieve an optimum range of tyrosine bonds in the dough.

33. The method of claim 32, further including the step of manipulating the range of tyrosine bonds within said dough.

34. The method of claim 32, further including the step of stopping the mixing when the predetermined optimum range is reached.

35. The method of claim 32, wherein the periodic analyzing of the dough is done by fluorometry.

36. The method of claim 33, wherein said manipulation step is chosen from a group comprising adding an amount of oxidizing agent to the dough, adding an amount of metal chelating agent to the dough, adding an amount of free tyrosine or tyrosine analogs to the dough, adjusting the pH of the dough, altering the mixing time, adding a free radical scavenger, and combinations thereof.

37. The method of claim 33, wherein the manipulation of the number of tyrosine bonds is accomplished by adding an oxidizing agent to the dough.

38. The method of claim 37, wherein said oxidizing agent is ascorbic acid.

39. The method of claim 37, wherein said oxidizing agent is $KBrO_3$.

40. The method of claim 33, wherein the manipulation of the number of tyrosine bonds is accomplished by adding free tyrosine and/or analogs thereof to the dough.

41. The method of claim 33, wherein the manipulation of the number of tyrosine bonds is accomplished by adding a metal chelating agent to the dough.

42. The method of claim 33, wherein the manipulation of the number of tyrosine bonds is accomplished by adjusting the pH of the dough.

43. The method of claim 33, wherein said manipulation of the number of tyrosine bonds in the dough is done in response to said comparison of said analyzed tyrosine range to said predetermined range.

44. The method of claim 43, wherein said manipulation of the number of tyrosine bonds in the dough is controlled by a computer program configured to achieve the desired range of tyrosine bonds by directing said manipulation in response to said analysis.

45. The method of claim 32, wherein said tyrosine bonds analyzed include tyrosine bonded with a moiety selected from a group comprising tyrosine, quinones, hydroquinone, dihydroxyphenylalanine (DOPA), dopaquinone, semiquinones, glutathione (GSH), cysteine, catechols, various carbohydrates and combinations thereof.

46. The method of claim 45, said tyrosine bonds being dityrosine bonds.

47. A method of analyzing flour to determine the approximate levels of tyrosine, dityrosine and/or phosphotyrosine contained therein comprising the steps of:

taking a sample of flour; and

analyzing said sample for said level(s).

48. The method of claim 47, further including the step of comparing said level(s) to a predetermined standard.

49. The method of claim 47, further including the step of precalibrating dough processing equipment based on said level(s).

50. The method of claim 47, wherein said analysis is performed by measuring the content of underivatized tyrosine in said flour.

51. The method of claim 47, wherein said analysis is performed by measuring the content of derivatized tyrosine in said flour.

52. The method of claim 47, wherein said analysis includes fluorometry.

53. The method of claim 47, wherein said analysis includes HPLC.

54. The method of claim 47, wherein said analysis includes determining the nucleic acid sequences of genes coding for glutenin and/or gliadin proteins in said flour.

55. A dough including the analyzed flour of claim 47.

56. A method for determining an ideal range of tyrosine bonds for a given stage of a dough production process comprising the steps of:

(a) preparing an ideal product using said production process;

(b) taking at least one sample during said production of said product; and

(c) analyzing the range of tyrosine bonds present in said sample for at least one stage of the production process.

57. The method of claim 56, wherein said analyzed range is designated as said ideal range for said stage.

58. The method of claim 56, wherein steps (a) -(c) are repeated a plurality of times for each said stage of said process.

59. The method of claim 58, wherein each said analyzed range for each said stage is averaged with the plurality of other analyzed ranges from corresponding stages from each other said repeated analysis in order to compute said ideal range for a specific stage from a plurality of samples.

60. A dough produced by the process of claim 32.

61. In a dough comprising admixed wheat flour and water, the wheat flour fraction of the dough having an inherent amount of tyrosine bonds formed therein when the dough during mixing thereof reaches a desired optimum condition, and wherein during continued mixing of the dough after said desired optimum condition is reached the amount of tyrosine bonds in the wheat flour fraction increases above said inherent amount to an undesirable amount, the improvement which comprises the addition of an additive which reduces further formation of tyrosine bonds in said wheat flour fraction of the dough during said continued mixing of the dough after said desired optimum is reached, as compared with said undesirable amount of tyrosine bonds.

62. The dough of claim 61, said additive comprising an amount of free tyrosine or tyrosine analogues admixed with said wheat flour and water in the dough.

63. A dough comprising admixed wheat flour and water and exhibiting a specific viscoelasticity at an optimum dough condition corresponding to an optimum dough mixing time, said dough exhibiting subsequent viscoelasticities during continued mixing of the dough for a continued mixing period of from about 5-20 minutes beyond said optimum dough mixing time, said subsequent viscoelasticities being within ± 20% of said specific viscoelasticity.

64. The dough of claim 63, said specific and subsequent viscoelasticities being measured by mixograph analyses according to mixograph method 54-40A of the American Association of Cereal Chemists.

65. The dough of claim 63, wherein said continued mixing period is up to about 10 minutes.

66. The dough of claim 63, said subsequent viscoelasticities being within ± 10% of said specific viscoelasticity.

67. The dough of claim 63, including a quantity of tyrosine bonds within said dough which has been altered as compared to the naturally occurring amount of tyrosine bonds.

68. A method of grading wheat comprising the steps of determining the level of tyrosine bonds in the glutenin fraction of the wheat, and using said level to predict the quality of the wheat.

69. The method of claim 68, including the step of determining said tyrosine bond level at a plurality of times during the growth of said wheat.

70. The method of claim 68, said determining said comprising the step of measuring the dityrosine bond level in said glutenin fraction.

71. A non-naturally occurring polymer comprising a polymer chain having a peptide within or attached to the polymer chain, said peptide selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14.

72. The polymer of claim 71, said polymer chain being a biopolymer.

73. The polymer of claim 72, said biopolymer selected from the group consisting of the plant proteins.

74. The polymer of claim 73, said plant proteins being selected from the group consisting of proteins of wheat, soy, corn, rye, oats, triticale, sorghum, rice, and barley.

75. The polymer of claim 73, said peptide being inserted within said polymer chain.

76. The polymer of claim 73, said peptide being attached to said polymer chain.

77. The polymer of 71, said polymer comprising a synthetic polymer.

78. A method of crosslinking the polymers of claim 71, comprising the steps of reacting a plurality of said polymers under conditions favoring crosslinking between the polymers and formation of tyrosine bonds therebetween.

79. The method of claim 78, including the step of carrying out said reaction in the presence of an agent selected from the group consisting of oxidizing agents, reducing agents, free radical generators, and free radical scavengers.

80. The method of claim 79, said oxidizing agent being selected from the group consisting of $KBrO_3$, ascorbic acid and ADA.

81. The method of claim 79, said reducing being selected from the group consisting of cysteine, glutathione, betamercaptoethanol and DTT.

82. The method of claim 79, said free radical generators being selected from the group consisting of the peroxides, peroxidases, and catalases.

83. The method of claim 79, said free radical scavengers being selected from the group consisting of BHT, cysteine, glutathione, and t-butyl catechol.

84. A method of growing a plant having one or more genes in the genome thereof which will express a protein containing a sequence selected from the group consisting of (1) peptides having the sequence X_aYYX_b; (2) peptides having the sequence $X_aQXGXYPTSX_b$; (3) peptides having the sequence $X_aQXGYXPTSX_b$; (4) peptides having the sequence $X_aGQGQXGXYPTSXQQX_b$; (5) peptides having the sequence $X_aGQGQXGYXPTSXQQX_b$, and (6) reversals of all of the foregoing, wherein each X independently represents any amino acid residue, and the sum of a + b ranges from 0-14, said method comprising the steps of periodically analyzing the plant or plant structures to determine the level of tyrosine bonds therein, and in response to such analysis applying a phosphate-containing nutrient to the plant or the soil adjacent the plant.

85. The method of claim 84, said plant being wheat, said analyzing step comprising the step of analyzing the endosperm of the wheat during growth thereof.

86. The method of claim 84, said applying step comprising the step of applying a phosphate fertilizer to the soil adjacent the plant.

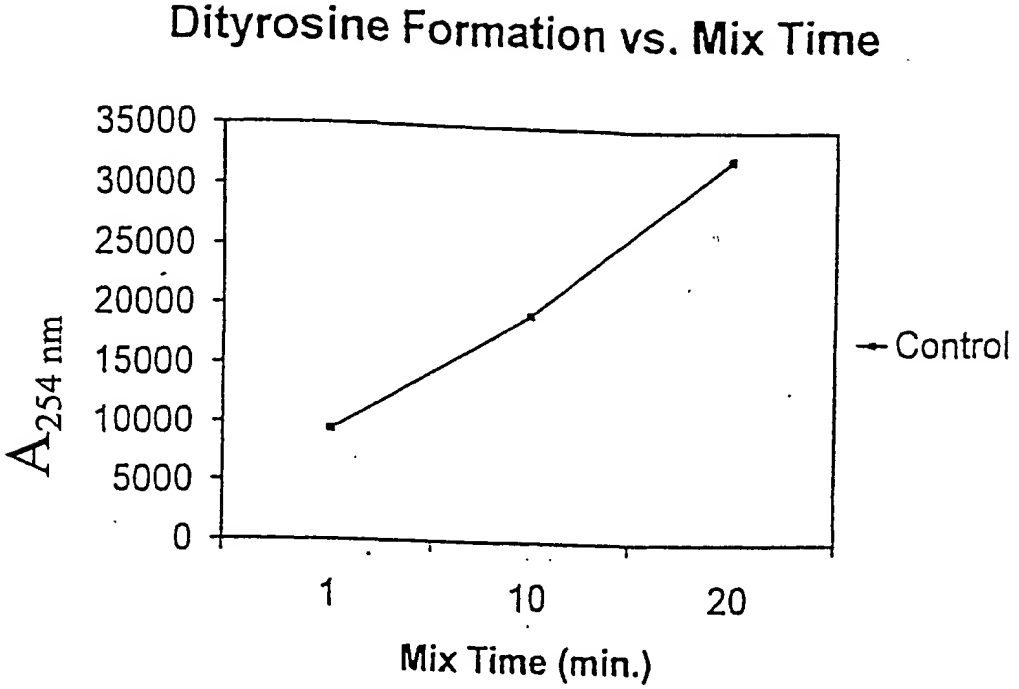

Dityrosine Formation vs. Mix Time
35000
30000
25000
20000
15000
10000
5000
0
A254 nm
1
10
20
Mix Time (min.)
Control

Fig. 1

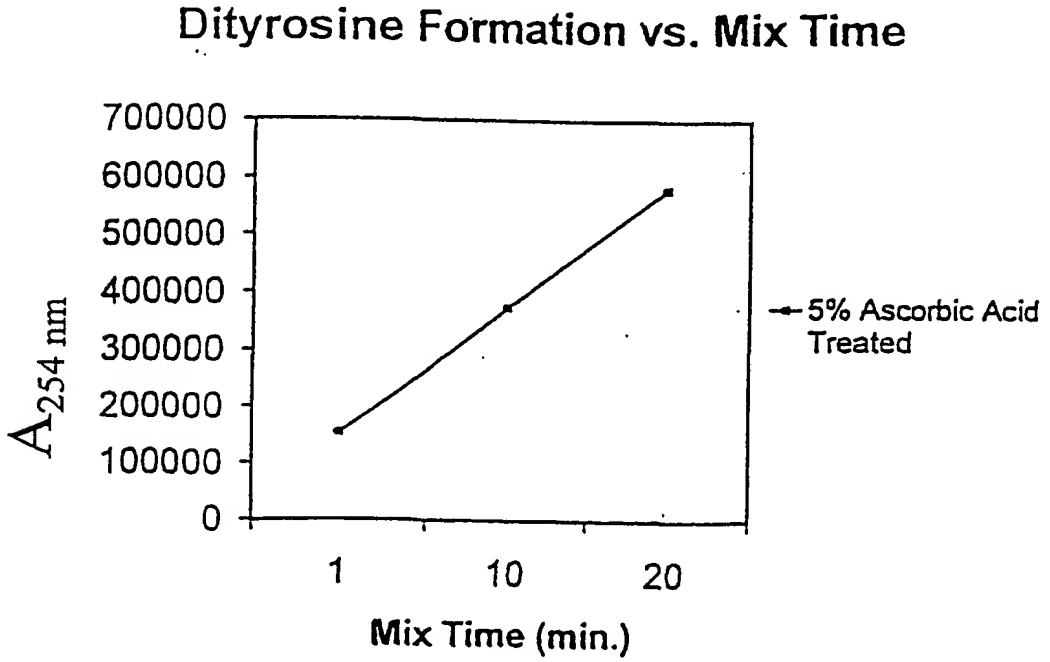

Dityrosine Formation vs. Mix Time
700000
600000
500000
400000
300000
200000
100000
0
A254 nm
1
10
20
Mix Time (min.)
5% Ascorbic Acid Treated

Fig. 2

Control Mixograph

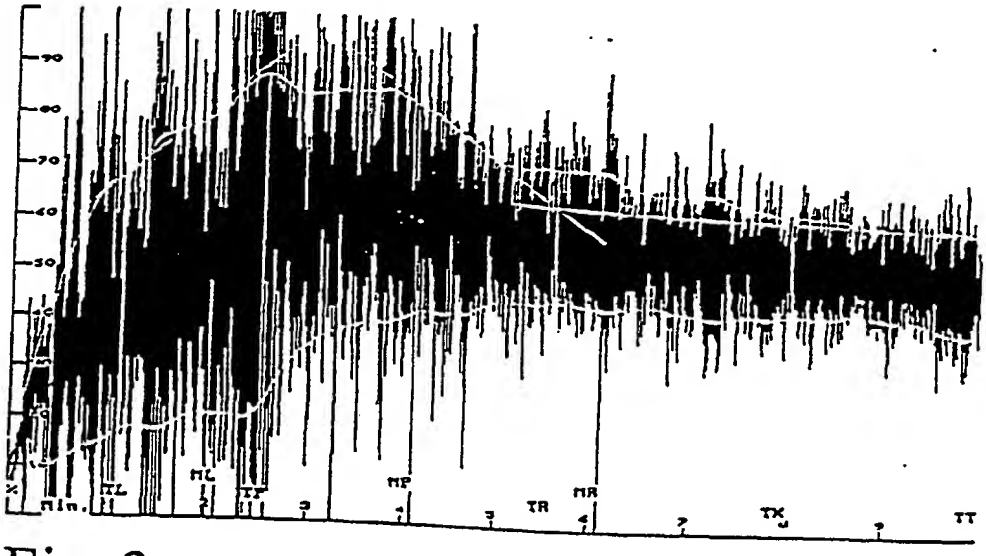


Fig. 3

1% Tyrosine

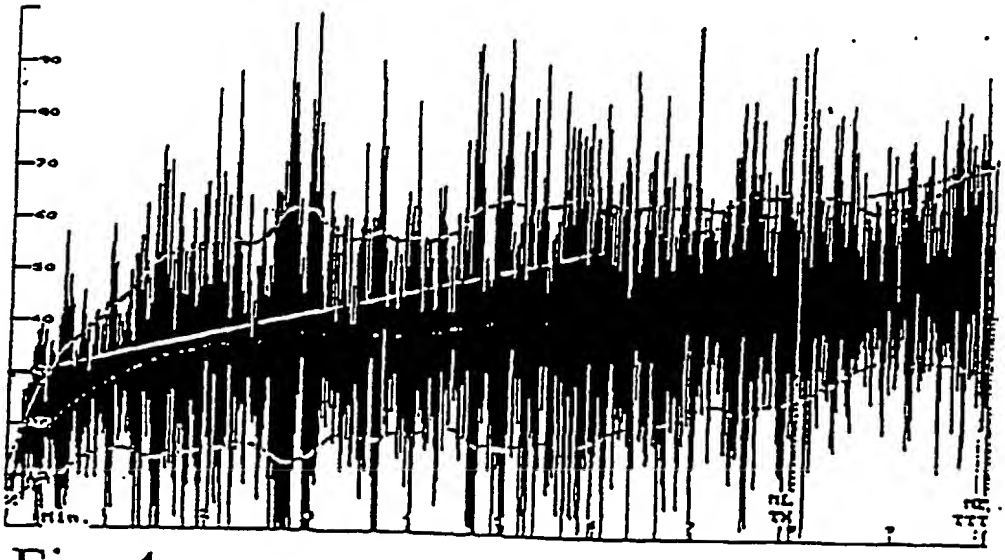


Fig. 4

1% Phosphotyrosine

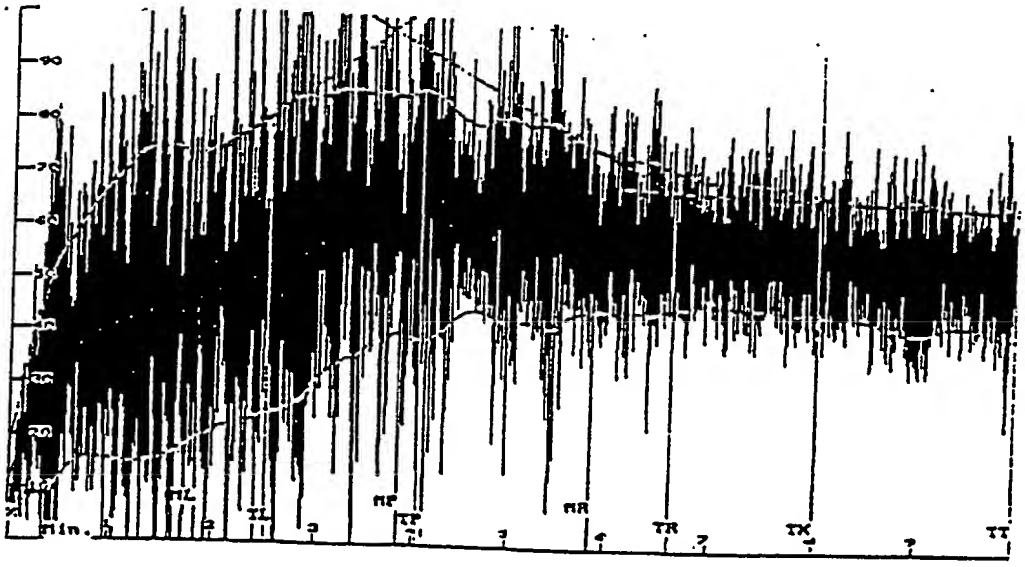


Fig. 5

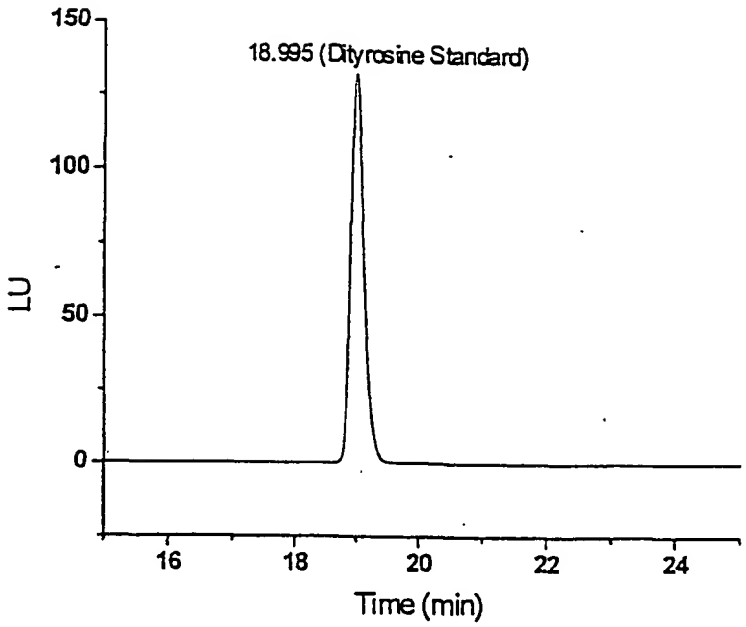


Fig. 6

Dityrosine Reference Standard

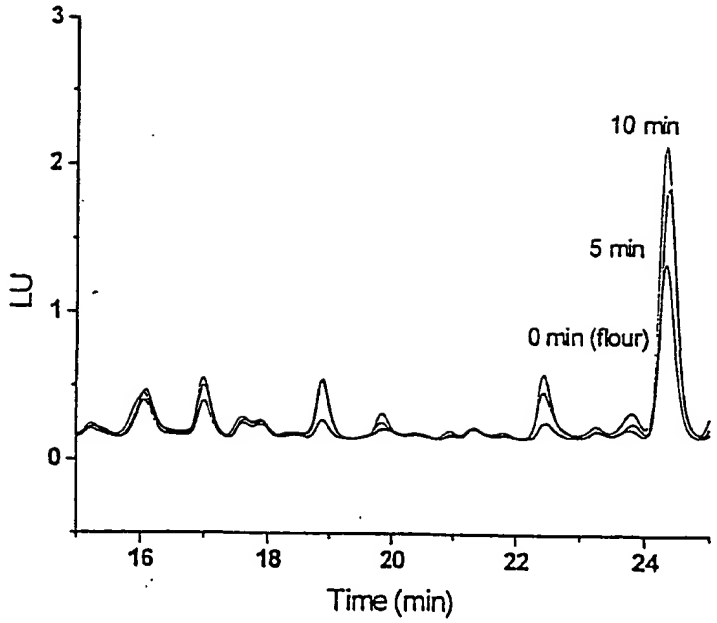


Fig. 7

Tyrosine Bonds Detected by Fluorescence

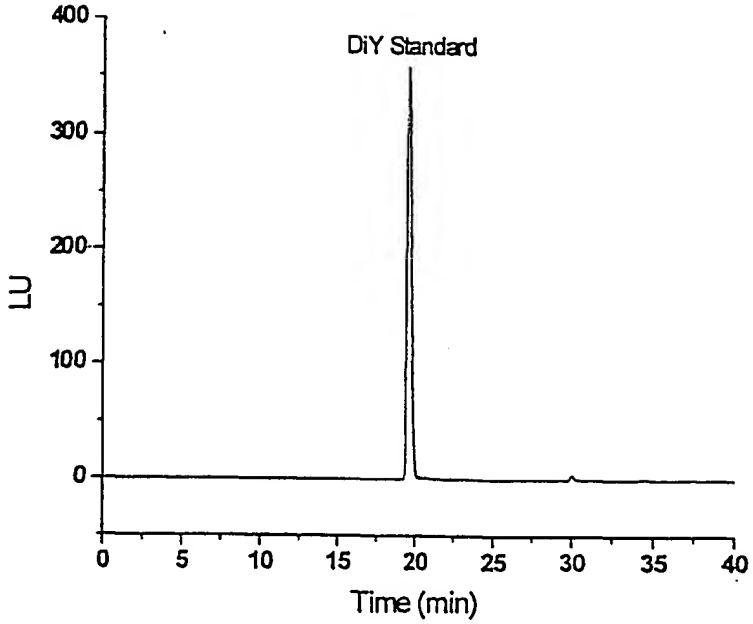


Fig. 8

Dityrosine Reference Standard

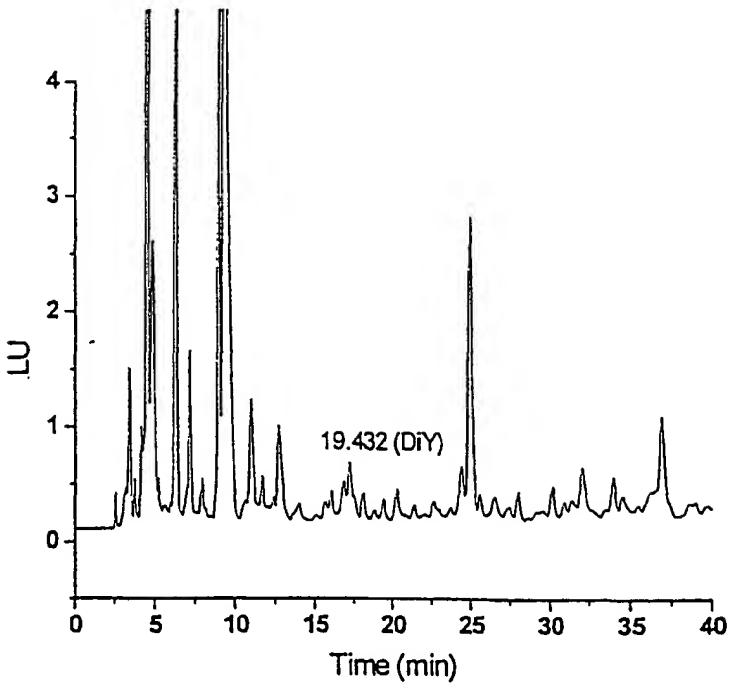


Fig. 9

Tyrosine Bonds Detected by Fluorescence

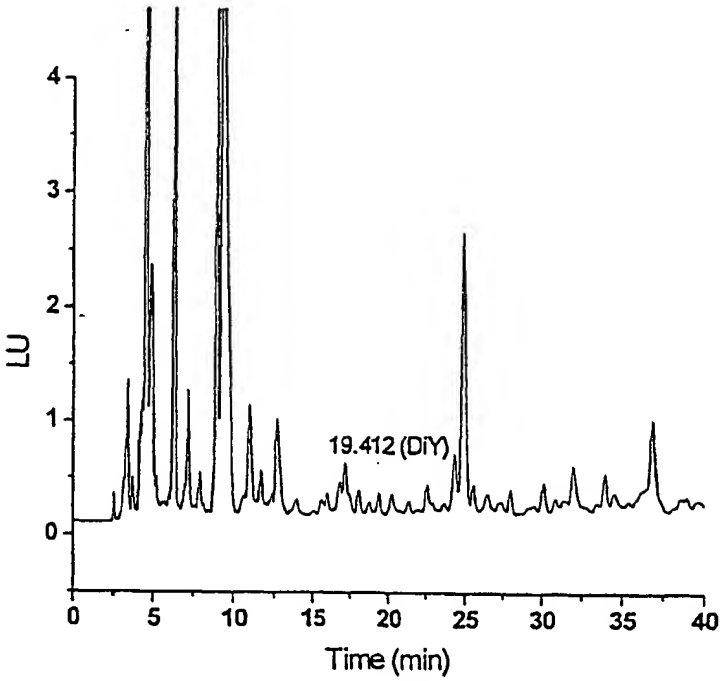


Fig. 10

Tyrosine Bonds Detected by Fluorescence

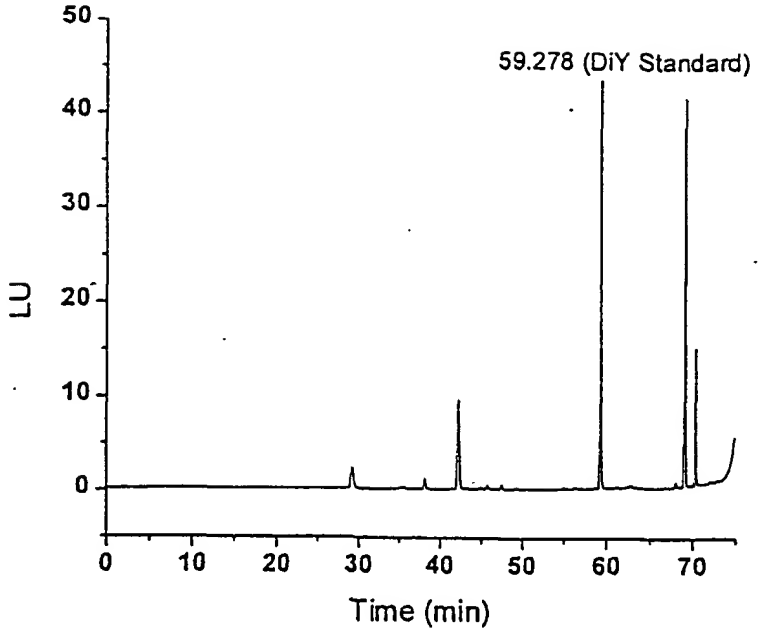


Fig. 11

Derivatized Dityrosine Reference Standard

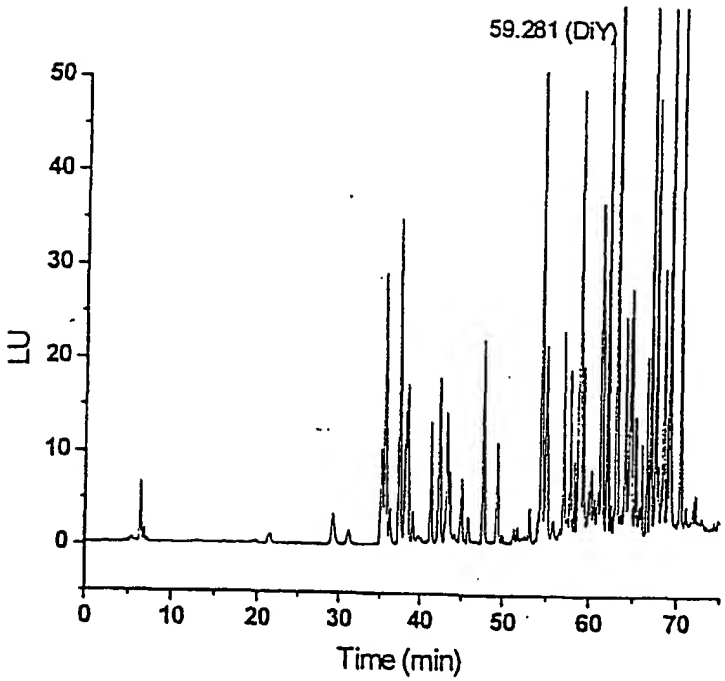


Fig. 12

Derivatized Tyrosine Bonds Detected by Fluorescence

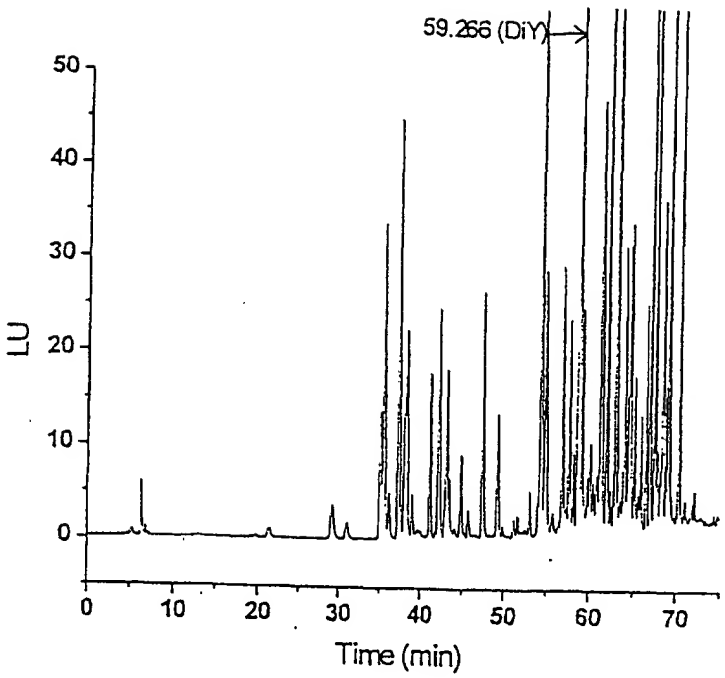


Fig. 13

Derivatized Tyrosine Bonds Detected by Fluorescence

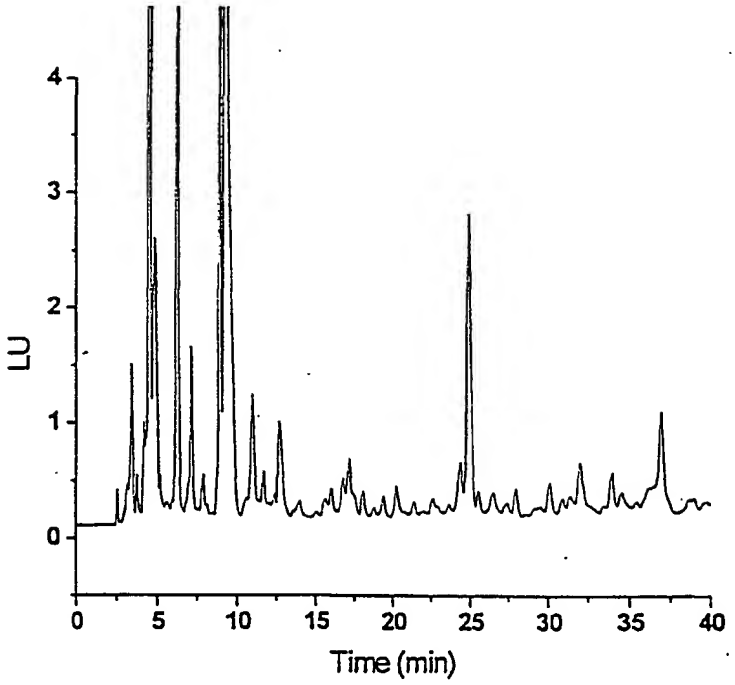


Fig. 14

Fluorescent Compounds Present in a Flour Sample

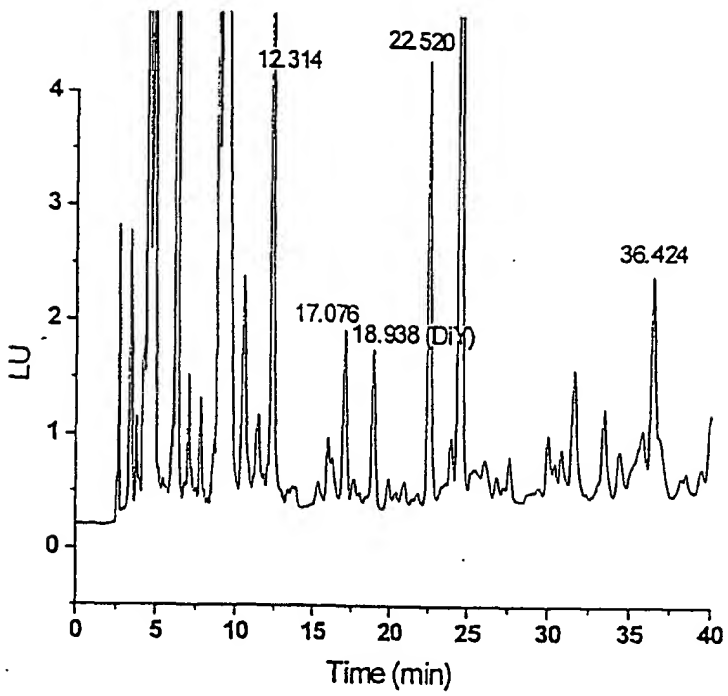


Fig. 15

Fluorescent Compounds of a Dough Sample

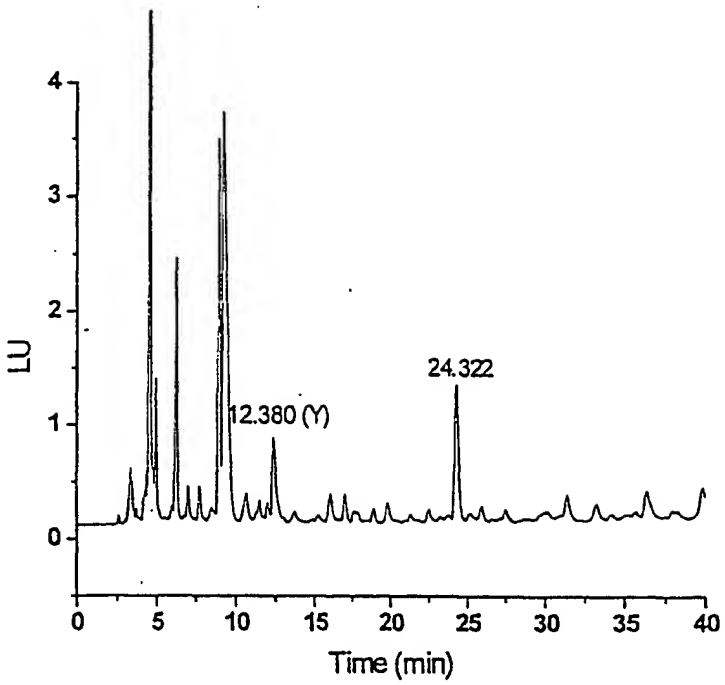


Fig. 16

Fluorescent Compounds Present in a Flour Sample

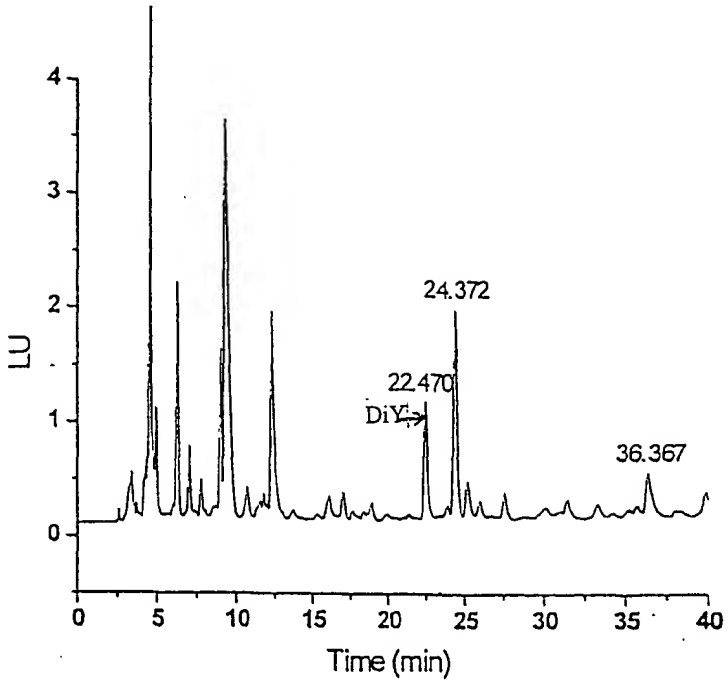


Fig. 17

Fluorescent Compounds in a Dough in the Presence of 1% Free Tyrosine After One Minute of Mixing

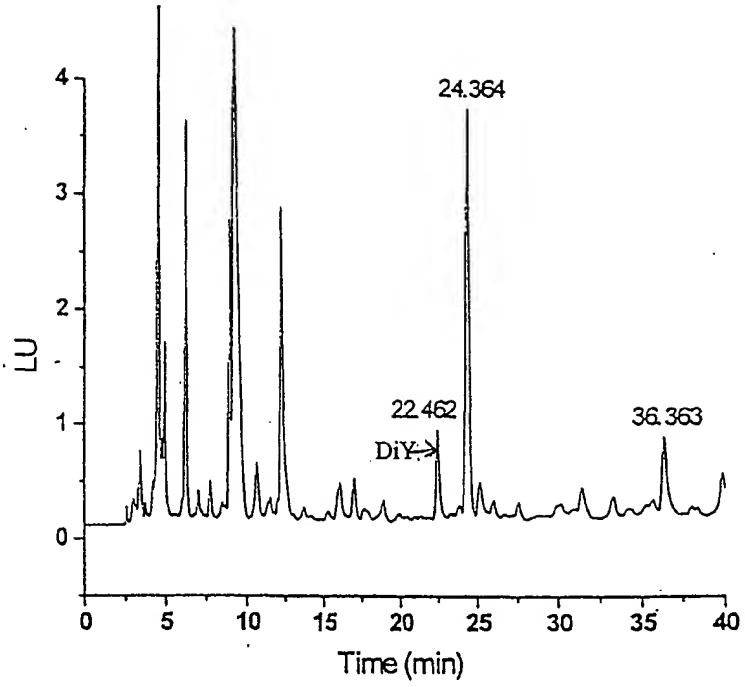


Fig. 18

Flourescent Compounds in a Dough in the Presence of 1% Free Tyrosine After Five Minutes of Mixing

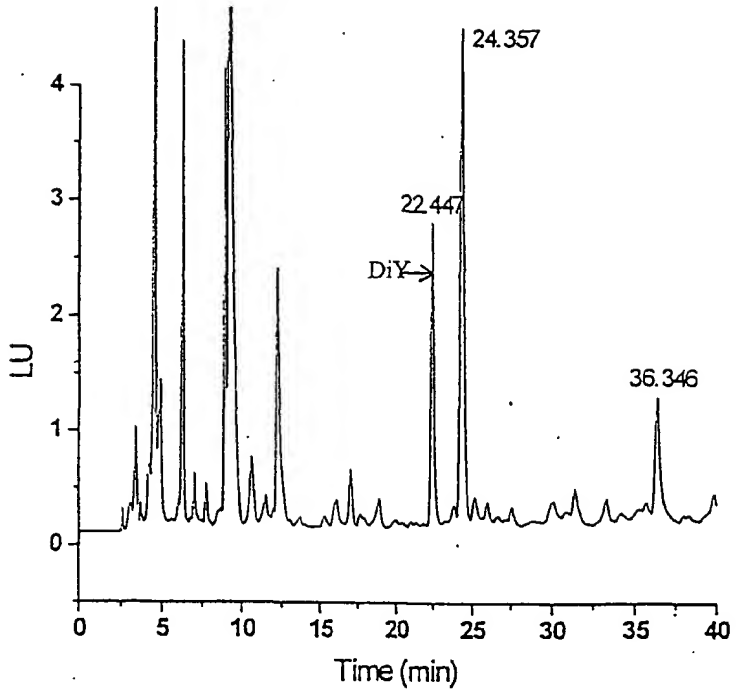


Fig. 19

Flourescent Compounds in a Dough in the Presence of 1% Free Tyrosine After Ten Minutes of Mixing

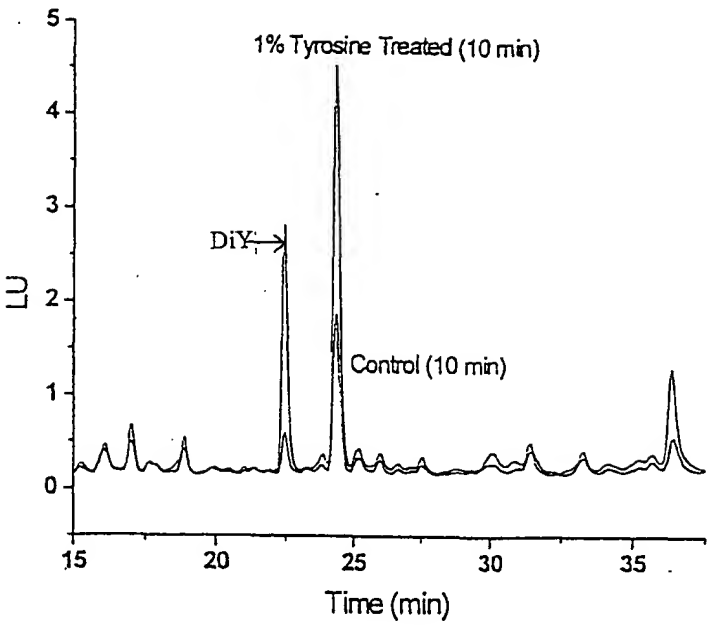


Fig. 20

Control Dough and Dough with 1% Free Tyrosine Added After Ten Minutes of Mixing

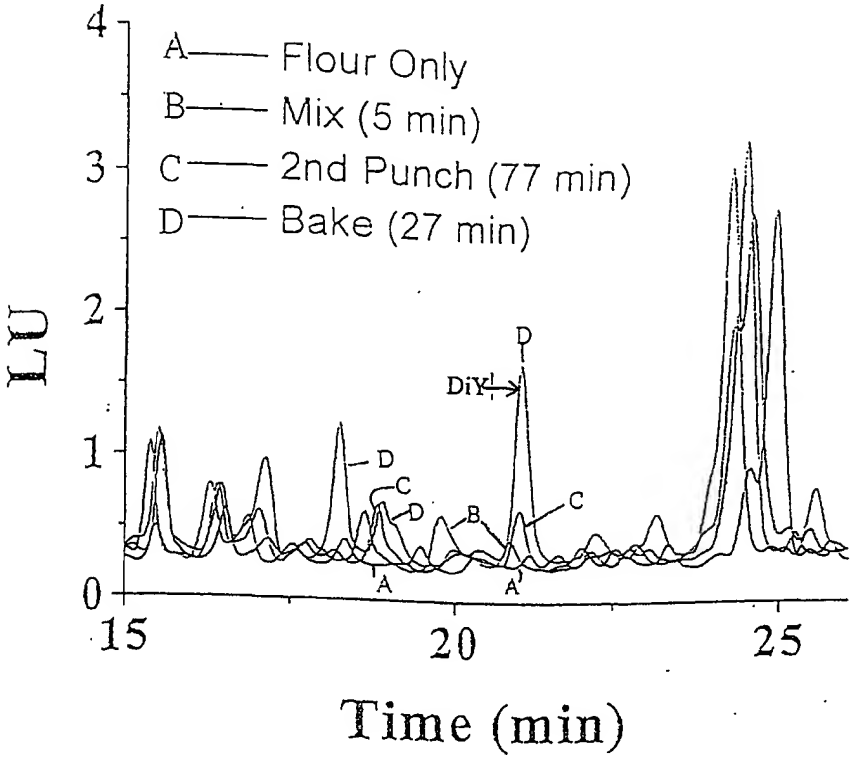

A—— Flour Only
B—— Mix (5 min)
C—— 2nd Punch (77 min)
D—— Bake (27 min)
LU
4
3
2
1
0
15
20
25
Time (min)
D
C
D
B
C
A
A'
D
Dif

Fig. 21

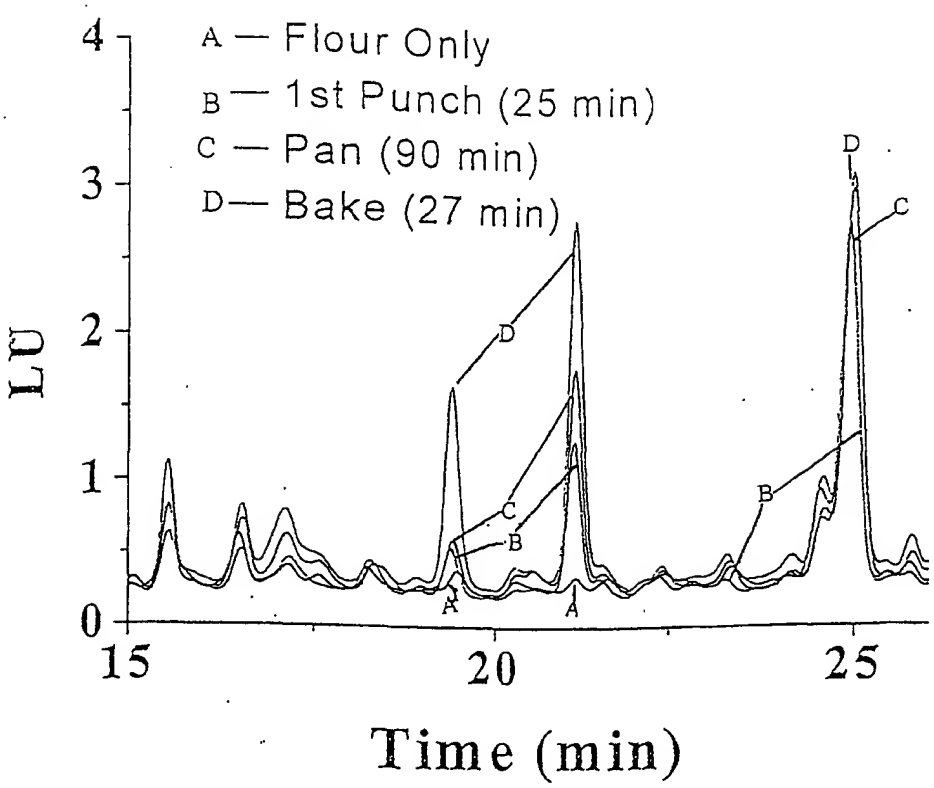

A — Flour Only
B — 1st Punch (25 min)
C — Pan (90 min)
D — Bake (27 min)
LU
4
3
2
1
0
15
20
25
Time (min)
A
B
C
D

Fig. 22

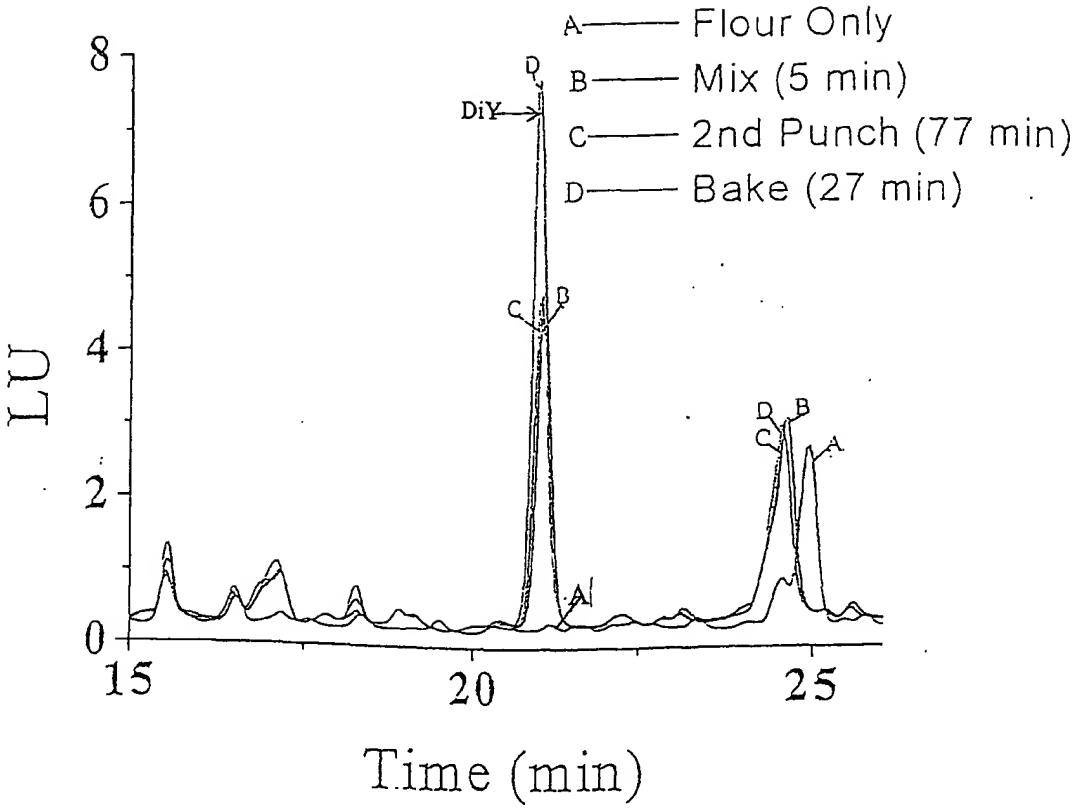

A—— Flour Only
B—— Mix (5 min)
C—— 2nd Punch (77 min)
D—— Bake (27 min)
LU
8
6
4
2
0
15
20
25
Time (min)
DiY
D
C
B
A

Fig. 23

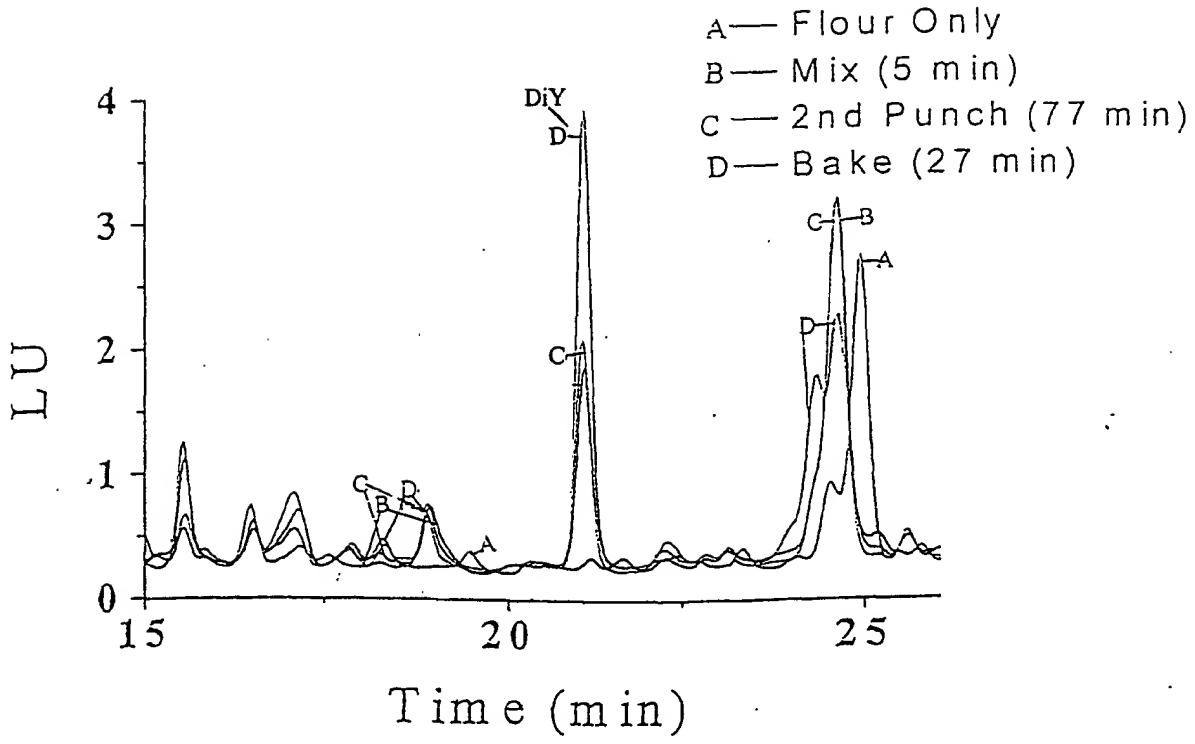

A— Flour Only
B— Mix (5 min)
C— 2nd Punch (77 min)
D— Bake (27 min)
LU
4
3
2
1
0
15
20
25
Time (min)
DiY
D
C
B
A

Fig. 24

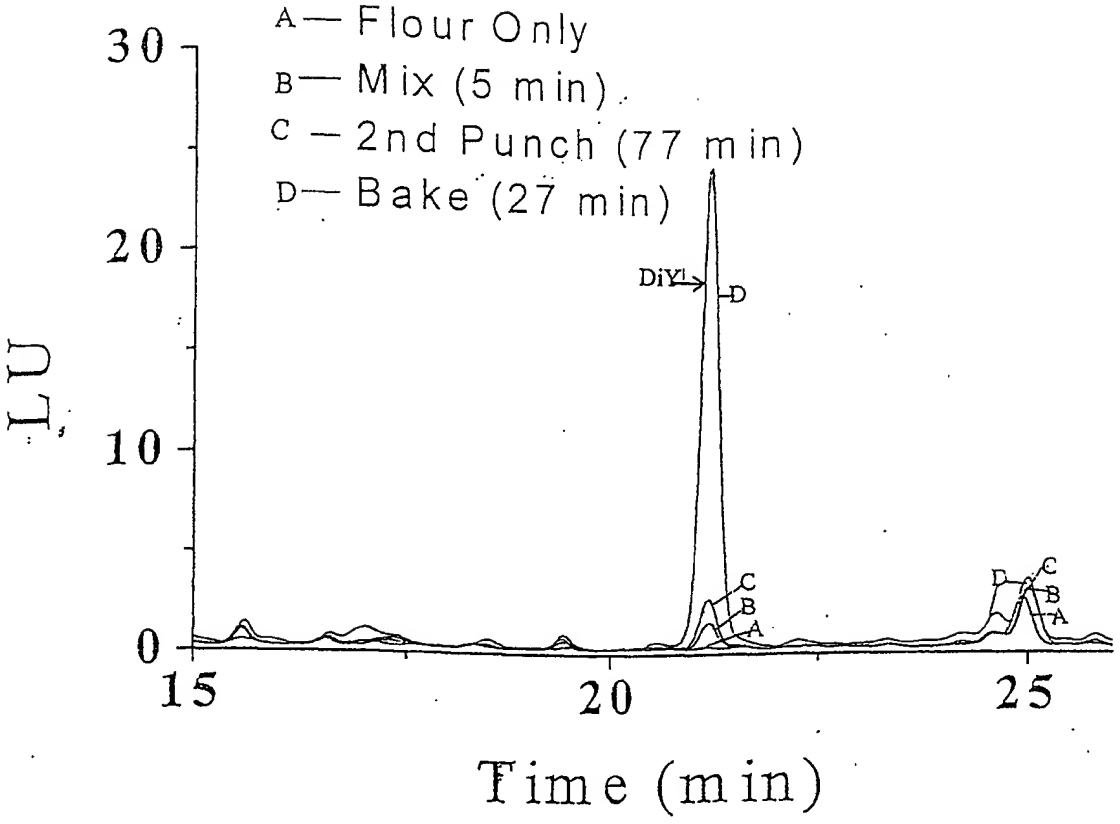

A— Flour Only
B— Mix (5 min)
C — 2nd Punch (77 min)
D— Bake (27 min)
DiVI
D
C
B
A
30
20
10
0
LU
15
20
25
Time (min)

Fig. 25

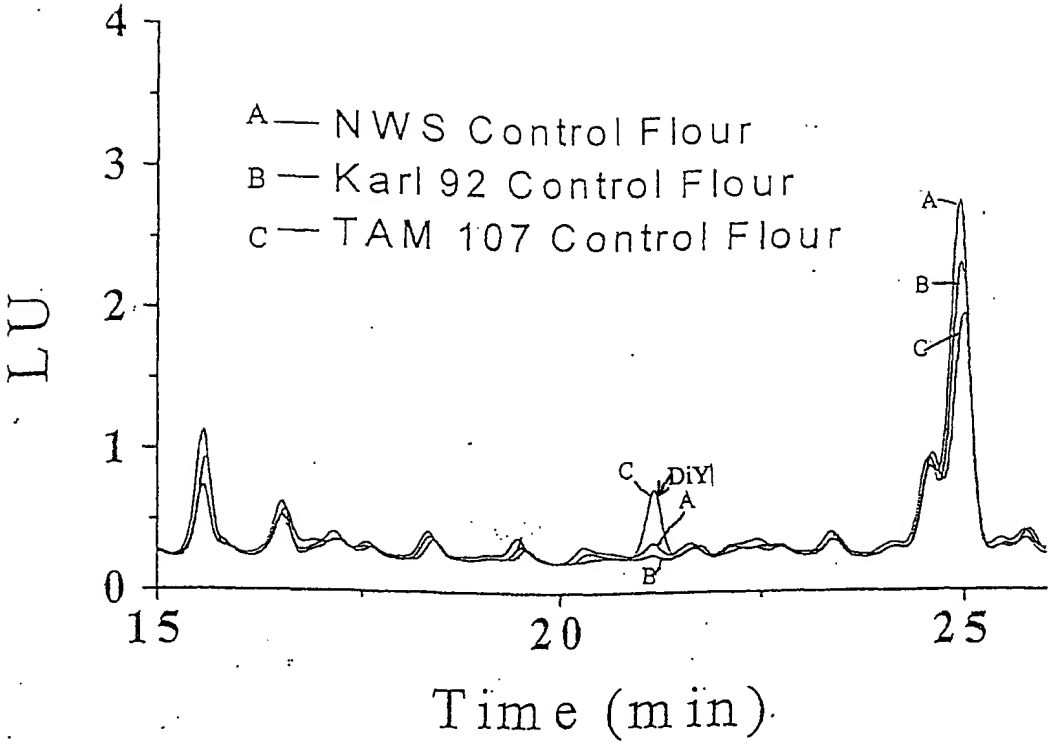

A— NWS Control Flour
B— Karl 92 Control Flour
C— TAM 107 Control Flour
LU
4
3
2
1
0
15
20
25
Time (min)
A
B
C
C
DiYl
A
B

Fig. 26

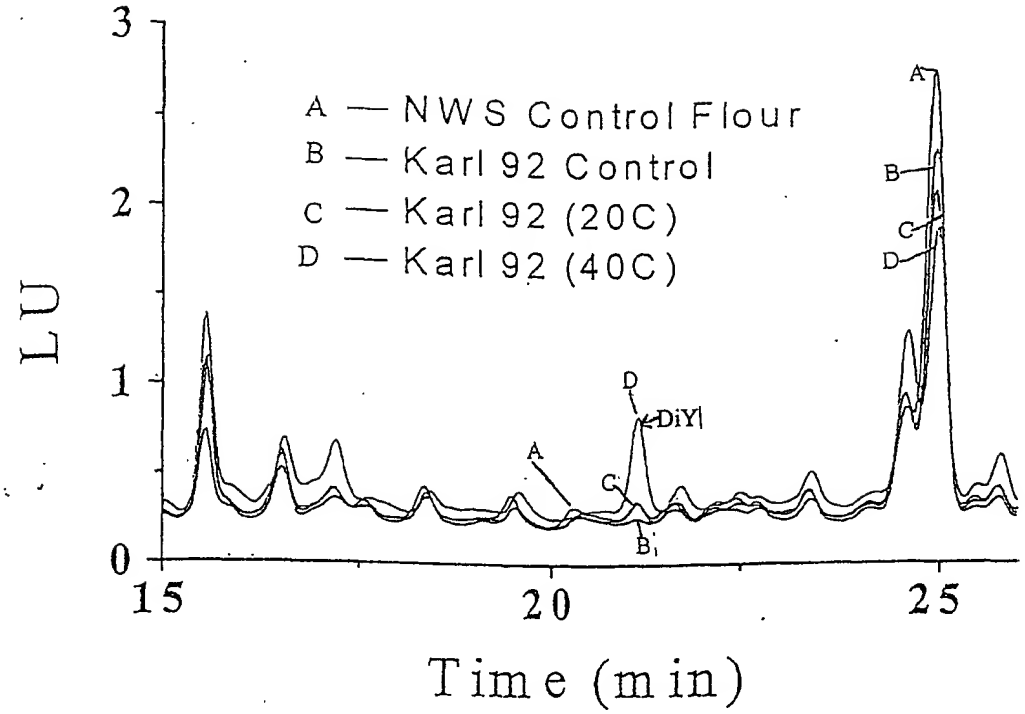

A — NWS Control Flour
B — Karl 92 Control
C — Karl 92 (20C)
D — Karl 92 (40C)
LU
Time (min)
3
2
1
0
15
20
25
A
B
C
D
DiY
Bi

Fig. 27

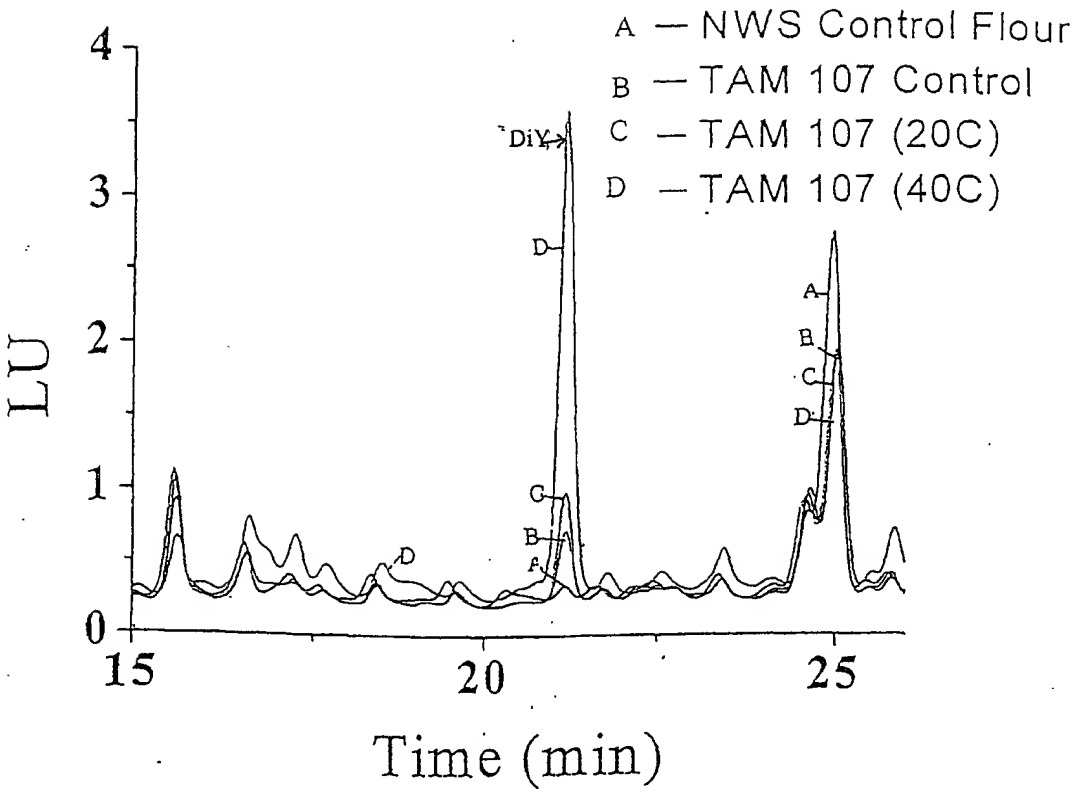

A — NWS Control Flour
B — TAM 107 Control
C — TAM 107 (20C)
D — TAM 107 (40C)
LU
Time (min)
0
1
2
3
4
15
20
25
DiV
A
B
C
D

Fig. 28

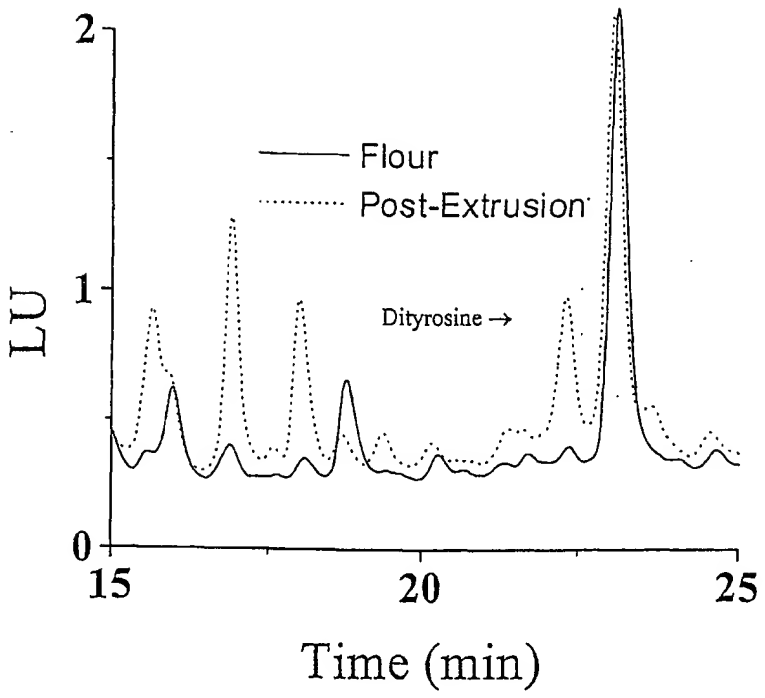


Fig. 29

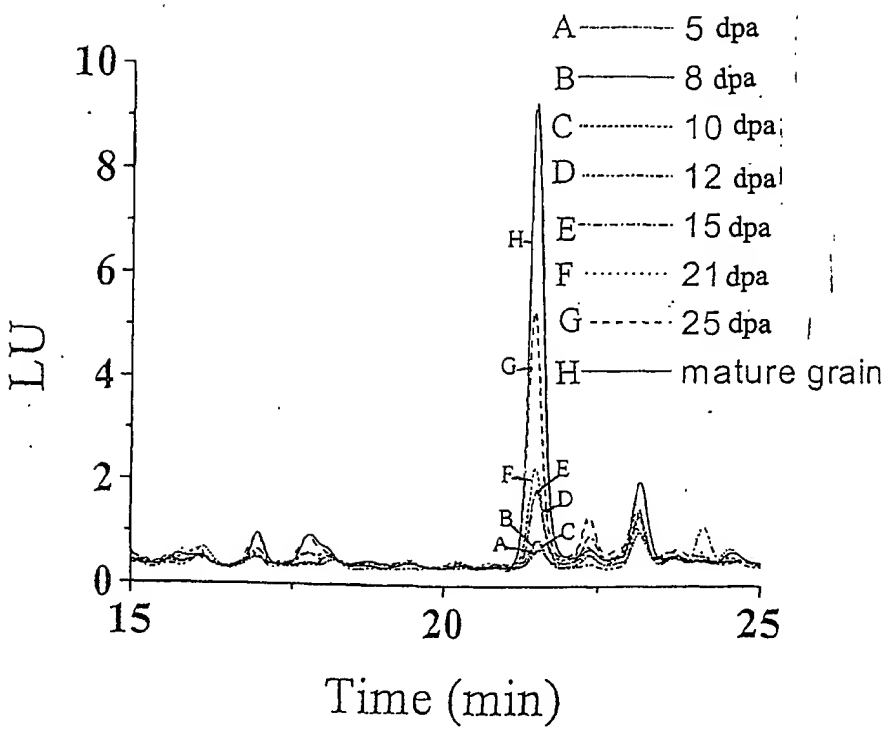

A 5 dpa
B 8 dpa
C 10 dpa
D 12 dpa
E 15 dpa
F 21 dpa
G 25 dpa
H mature grain
LU
10
8
6
4
2
0
15
20
25
Time (min)

Fig. 30

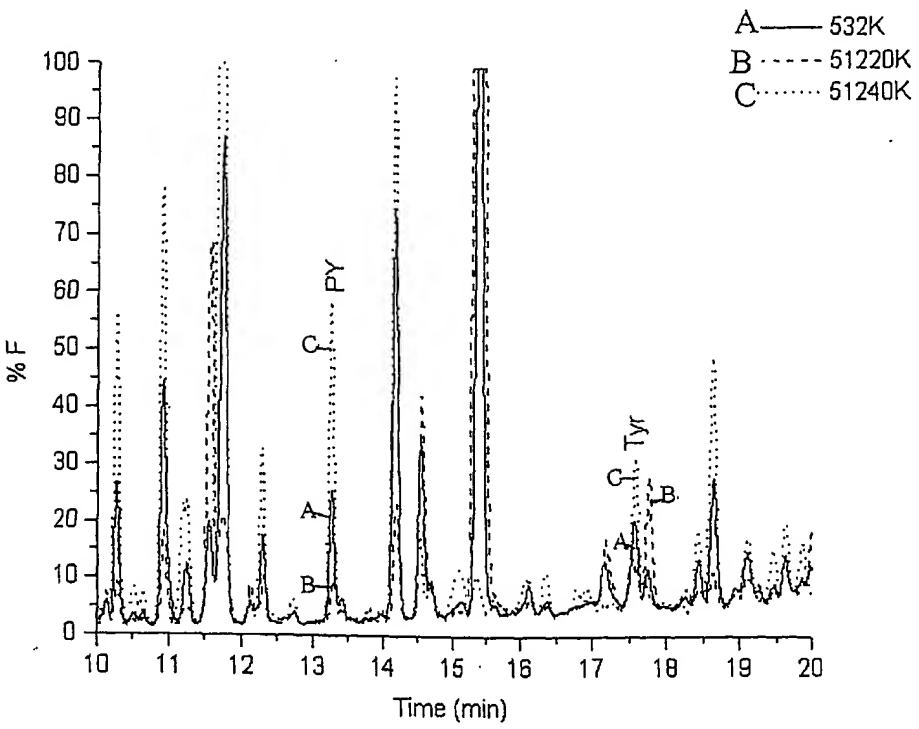


Fig. 31

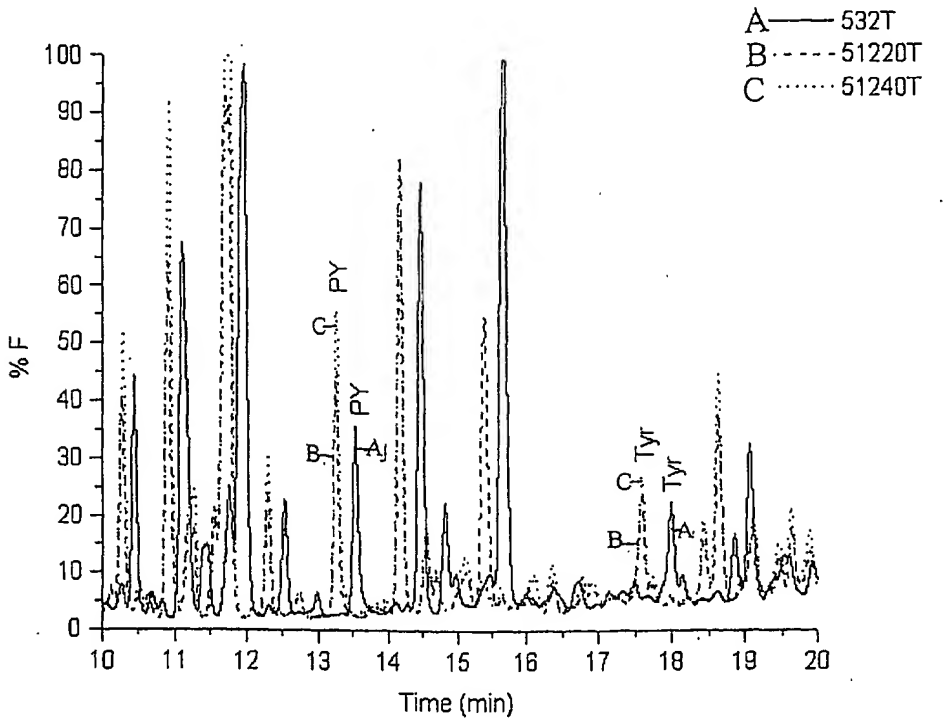

A—— 532T
B----- 51220T
C ····· 51240T
% F
100
90
80
70
60
50
40
30
20
10
0
10
11
12
13
14
15
16
17
18
19
20
Time (min)
PY
PY
Tyr
Tyr
A
B
C

Fig. 32

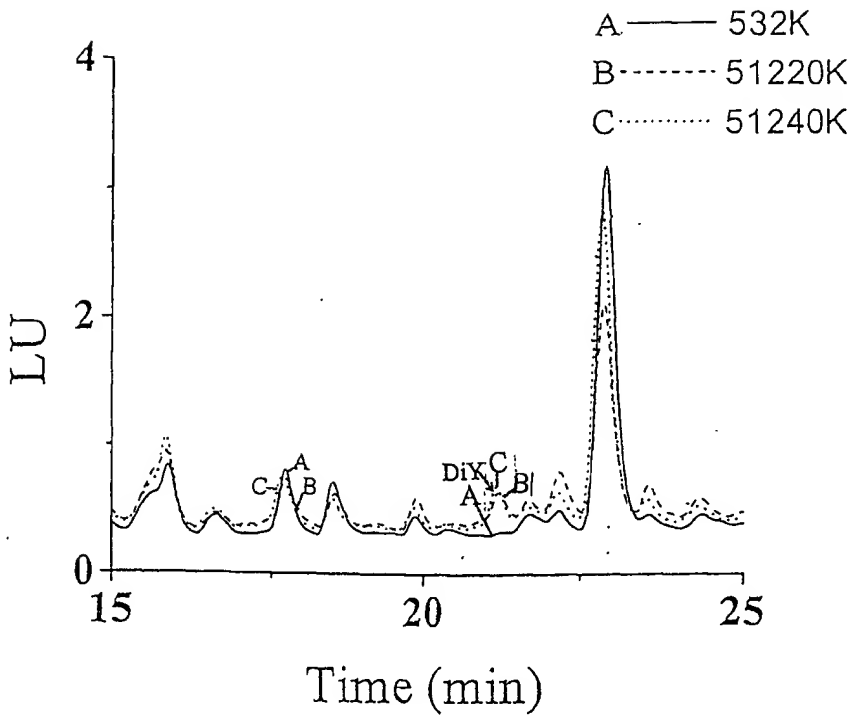


Fig. 33

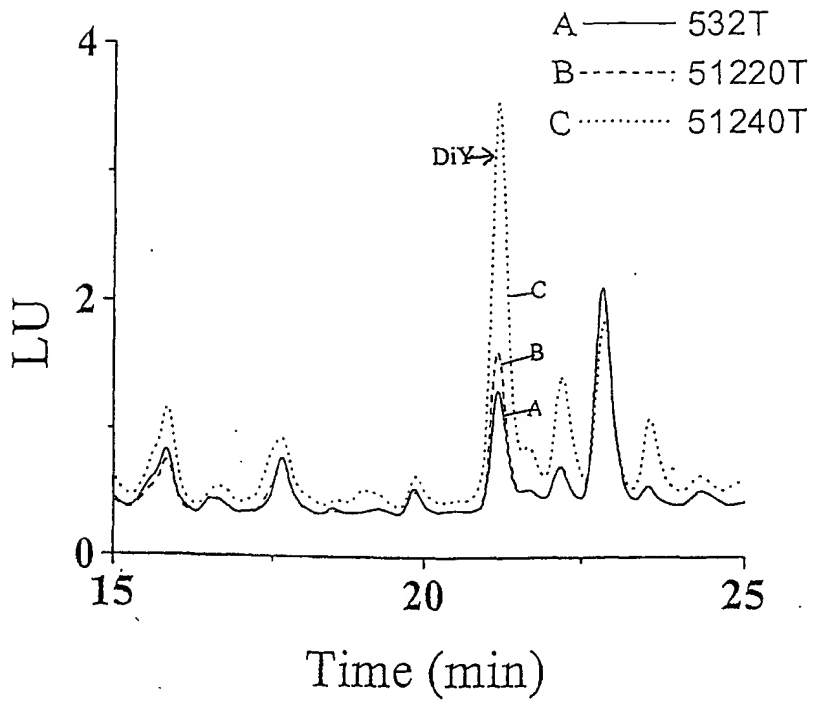


Fig. 34

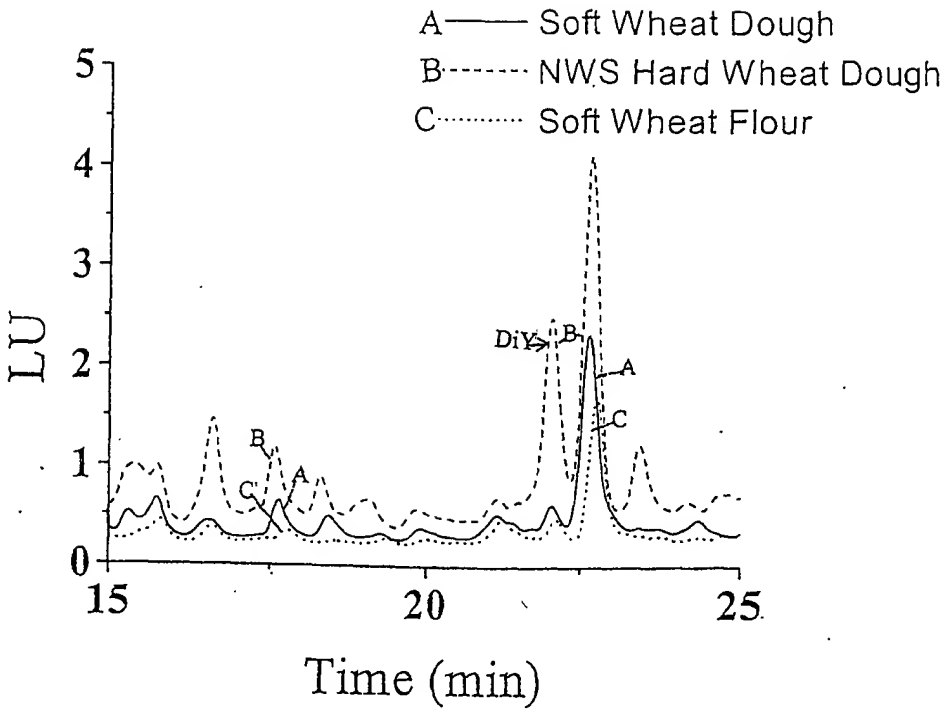

A—— Soft Wheat Dough
B------ NWS Hard Wheat Dough
C..... Soft Wheat Flour
LU
5
4
3
2
1
0
15
20
25
Time (min)
DiV
A
B
C

Fig. 35

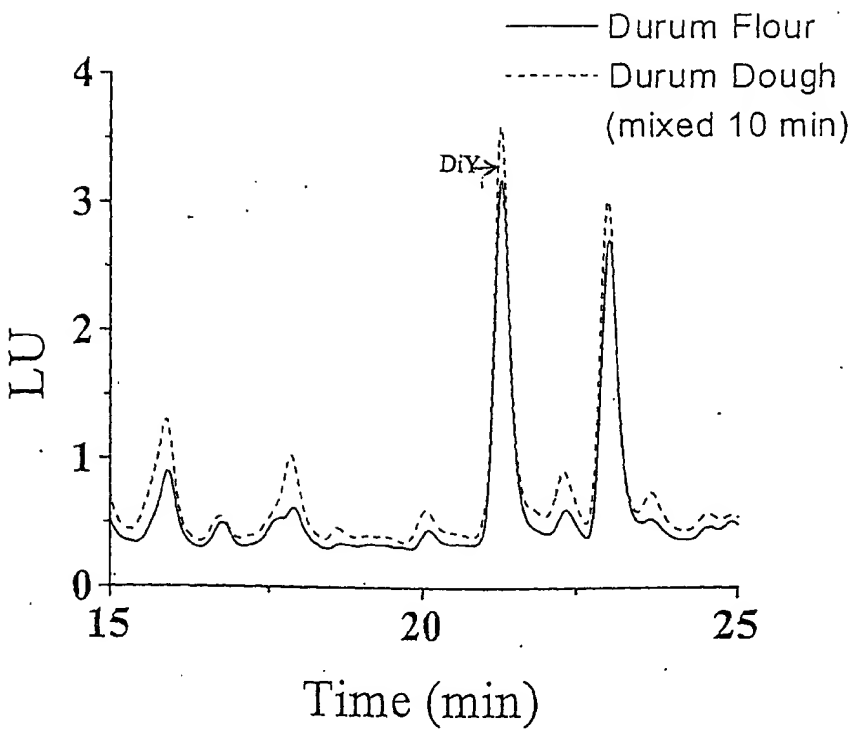

Durum Flour
Durum Dough
(mixed 10 min)
DiY
LU
4
3
2
1
0
15
20
25
Time (min)

Fig. 36

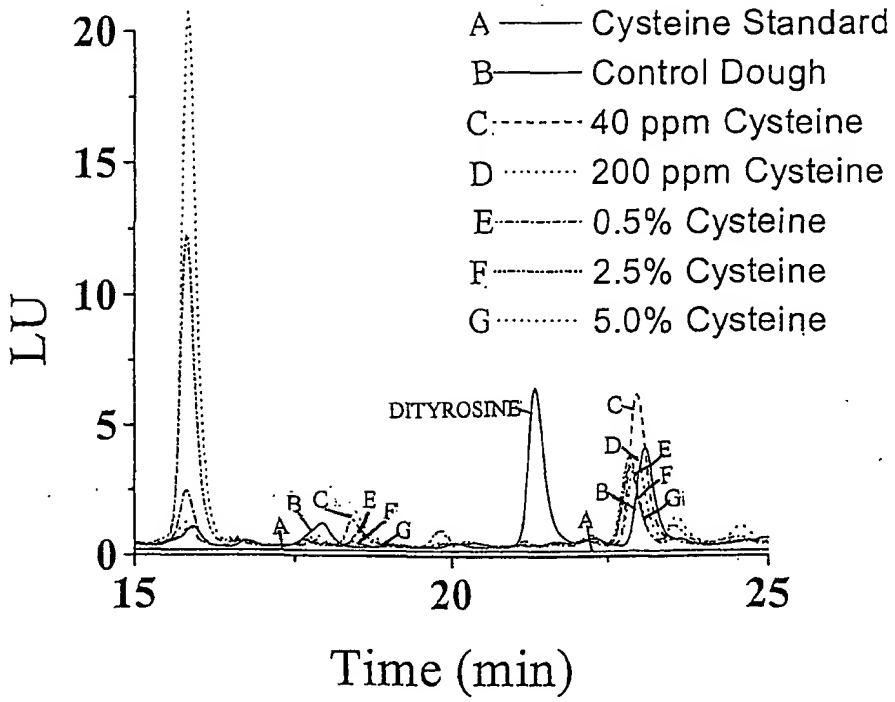

A —— Cysteine Standard
B—— Control Dough
C------ 40 ppm Cysteine
D ..... 200 ppm Cysteine
E------ 0.5% Cysteine
F------ 2.5% Cysteine
G..... 5.0% Cysteine
LU
20
15
10
5
0
DITYROSINE
15
20
25
Time (min)

Fig. 37

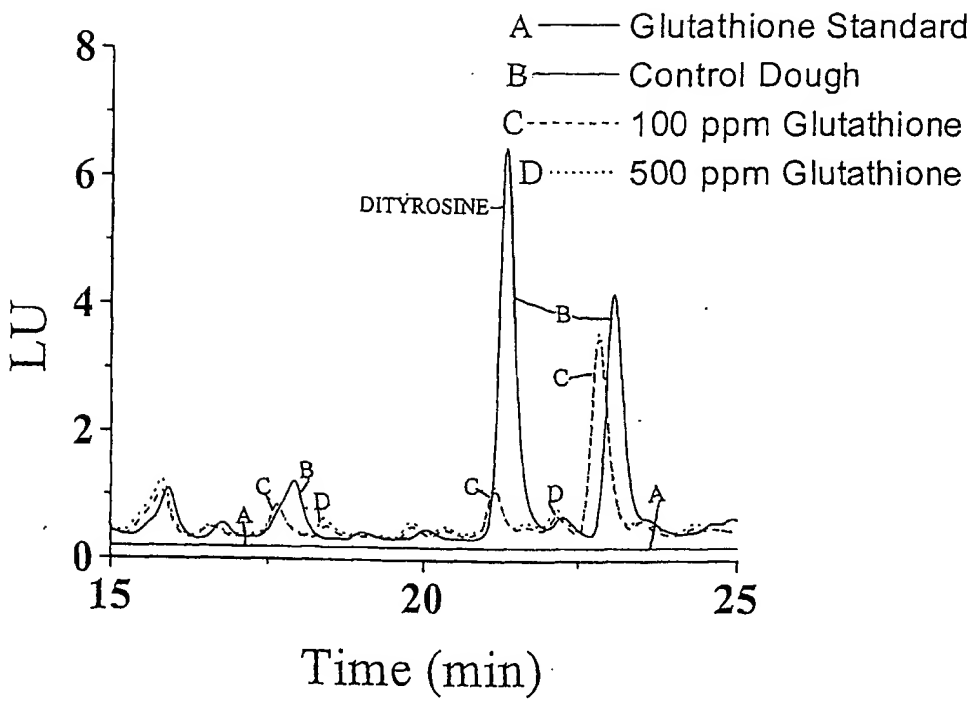

A —— Glutathione Standard
B —— Control Dough
C ------ 100 ppm Glutathione
D ..... 500 ppm Glutathione
DITYROSINE
LU
8
6
4
2
0
15
20
25
Time (min)

Fig. 38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02106

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01H 01/00;C12P 21/00;C07K 1/00,4/10,5/00,7/00,14/00;A21D 2/00,6/00,13/00; G01N 33/10
US CL : 800/295;435/69.1, 69.7, 410;530/370,333;426/391,496,549,622,231

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 426/231,271,391,496,504,549,622;800/295,320.3;435/69.1,69.7,410;935/51,64;530/300,333,350,370,374,375

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 98/08607 A1 (ANDERSON) 05 March 1998 (05.03.1998), entire disclosure, especially the examples on pages 15 and 16.	1,4-7,9, 71-74 --- 2,3,8,10-27,40, 75-79,82,84-86
Y	WO 98/48271 A1 (WESLEY, et al) 29 October 1998 (29.10.1998), entire document.	22-46,55-67,75-79,80-8382
Y	MICHON,et al. Horseradish Persoxidase Oxidation of Tyrosine -Containing Peptides and Their Subsequent Polymerization: A Kinetic Study Biochemistry. 28 November 1997, Vol. 36, p.8504-8513	10,16,18,19,21-46,55-67,75-79,82
Y	US 4,135,816 A (NIEMANN et al.) 23 January 1979 (23.01.1979), especially columns 4 and 8.	28-70
Y	US 5,510,129 A (KIM) 23 April 1996 (23.04.1996), especially claims and column 1, lines 17-44.	36-39,41-42,80
Y	WO 99/03985 A1 (BLECHL et al.) 28 January 1999 (28.01.1999), entire document.	47-55,68-70,80,81,83

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
	14 JUN 2000

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Robert Madsen
Facsimile No. (703)305-3230	Telephone No. (703)308-0651

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02106

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
I. Claims 1-21, 71-86, drawn to peptides and the method of making pepetide sequences.
II. Claims 22-27, drawn to a method of altering a protein by altering a gene.
III. Claims 28-67, drawn to a dough and a method of making dough.
IV. Claims 68-70, drawn to a method of grading wheat.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.